

MORPHINE UNDERMINES RECOVERY FOLLOWING SPINAL CORD INJURY:  
EVALUATION OF OPIOID RECEPTORS AND CELLULAR MECHANISMS

A Dissertation

by

MIRIAM ACEVES

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Chair of Committee,  
Committee Members,

Michelle A. Hook  
James W. Grau  
Rajesh Miranda  
Ursula Winzer-Serhan

Interdisciplinary Faculty  
Chair,

Jane Welsh

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## ABSTRACT

Opioids are amongst the most effective and widely prescribed medications for the treatment of pain following spinal cord injury (SCI). Spinally-injured patients receive opioids shortly upon arrival at the emergency room, and prolonged opioid regimens are often employed for the management of post-SCI chronic pain. However, despite their utility, questions remain regarding the safety of these analgesics in the clinical setting. Indeed, previous studies in our laboratory suggest that the effects of opioids, such as morphine, may be altered in the pathophysiological context of neurotrauma. Specifically, our studies suggest that morphine use is contraindicated in the early phase of SCI. In our rodent model of SCI, acute morphine treatment increases tissue loss at the injury site, increases mortality, undermines weight gain and reduces recovery of motor and sensory function even weeks after treatment. The literature also suggests that opioids may exacerbate secondary injury mechanisms following SCI, increasing neurotoxicity at the lesion site. Based on these data, it would be tempting to suggest that morphine be eliminated as an analgesic after SCI. However, as pain is one of the most debilitating consequences of SCI, we cannot afford to simply remove a potential analgesic therapy. To address this, the experiments presented here examined the molecular mechanisms underlying the adverse effects of morphine. The first set of experiments evaluated classic opioid receptor signaling and showed that the  $\kappa$ -opioid receptor (KOR) system significantly contributes to the morphine-induced attenuation of function following SCI. The second set of experiments showed that, while non-classic

opioid receptor signaling is not sufficient, glial activation is critical to morphine's adverse effects. The last set of experiments investigated the effects of acute morphine treatment on the cellular environment of the lesion site. The findings suggest that opioid-immune interactions following SCI – mediated by the KOR system – may exacerbate secondary injury mechanisms, leading to increased cell death and attenuated recovery of function. Together, the results presented here underscore the need to improve the safety and efficacy of opioids in the clinical setting.

## DEDICATION

Porque cada éxito en mi vida es testimonio de tus sacrificios, tu apoyo incondicional, y tu amor infinito, esto te lo dedico a ti.

Gracias, mamá.

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# CHAPTER I

## INTRODUCTION AND LITERATURE REVIEW

### **Pain following spinal cord injury**

Pain is one of the most debilitating consequences of spinal cord injury (SCI). Approximately two-thirds of spinally-injured patients develop chronic pain within 5 years of their injury (Gorp et al., 2015; Siddall & Loeser, 2001; Siddall et al., 2003). Along with recovery of motor, bladder/bowel, and sexual function, patients often rate pain management as one of their top priorities following SCI (Anderson, 2004; Simpson et al., 2012). Importantly, chronic pain has been associated with diminished physical and psychological health, disrupted sleep patterns, impaired social interactions and community involvement, and an overall decrease in vitality and well-being (Attal et al., 2011; Gormsen et al., 2010; Jensen et al., 2007; McDermott et al., 2006; Smith et al., 2007). Clearly, effective pain management is imperative to improve quality of life for patients suffering from a spinal cord injury.

Treatment of post-SCI chronic pain, however, has been complicated by its complex etiology and heterogeneity. Although a number of anatomical and neurochemical alterations have been identified as contributing to chronic pain following SCI (Finnerup & Jensen, 2004; Hassler et al., 2014; Lee-Kubli et al., 2016; Lerch et al., 2014; Q. Yang et al., 2014), the precise mechanisms underlying its initiation and maintenance remain to be elucidated. Clinically, spinally-injured patients often describe a wide range of chronic pain symptoms, including dull, aching, sharp, shooting,

stabbing, electric, or even burning sensations (Siddall et al., 2003). Variations in intensity (mild to severe pain), duration (persistent versus intermittent), and responsiveness to stimulation (spontaneous versus evoked) further complicate management of chronic pain. In addition, exacerbating the frustration of patients and health-care professionals, chronic pain often shows resistance to conventional therapeutic strategies (Warms et al., 2002). Consequently, treatment of chronic pain following SCI has been based on a trial-and-error approach.

Opioids are among the most potent analgesics currently available, and have shown efficacy in the management of chronic pain conditions (Eisenberg et al., 2006; Gilron et al., 2005; Gimbel et al., 2003; Raja et al., 2002; Warms et al., 2002; Watson et al., 2003). However, due to concerns about unwanted side effects, opioids are no longer recommended as a first-line medication for the treatment of chronic pain (Attal et al., 2010; Eisenberg et al., 2005; Furlan et al., 2006; Kalso et al., 2004; O'Connor & Dworkin, 2009). Indeed, many unwanted effects associated with opioid administration have been well documented (Buenaventura et al., 2008; McNicol et al., 2003). Some of the side effects that can result from acute opioid treatment include nausea, itching, vomiting, dizziness, drowsiness, and constipation. Respiratory depression can also occur at high doses. Opioids can also interact with the endocrine system, resulting in hormone suppression, hypogonadism, and altered sexual function (Abs et al., 2000; Daniell, 2002; N. Katz & Mazer, 2009). Additionally, with prolonged use, opioid tolerance, dependence, and addiction can develop (Chu et al., 2006; Højsted & Sjøgren, 2007; Martell et al., 2007). Paradoxically, long term administration of opioids has also been

linked to the development of abnormal pain sensitivity, an effect known as opioid-induced hyperalgesia (Angst & Clark, 2006; Chu et al., 2008; Chu et al., 2006).

Alarming, evidence also suggests that opioids may be contraindicated following injury to the spinal cord (Hook et al., 2007; Hook et al., 2009; Hook et al., 2011; Woller et al., 2014; Woller et al., 2012). Using a rodent model of SCI, our previous studies have shown that regardless of the route of administration (intraperitoneal, intrathecal, or intravenous), morphine given in the acute phase of SCI increases tissue loss at the injury site, increases mortality, undermines weight gain, reduces recovery of motor and sensory function, and increases pain reactivity, even weeks after treatment (Hook et al., 2007; Hook et al., 2009; Hook et al., 2011; Woller et al., 2014; Woller et al., 2012). Based on these data, it may be tempting to eliminate opioids from the list of treatments currently used for post-SCI pain. However, for patients facing a lifetime of intractable pain, simply removing these powerful drugs from clinical use is not an option. Instead, further understanding of the interactions between opioids and injury is necessary to improve the safety and efficacy of these drugs.

### **Mechanisms of opioid-induced cell death**

Elucidating the molecular mechanisms underlying the adverse effects of opioids is necessary to improve the safety and efficacy of these drugs. The dramatic consequences of morphine administration following SCI (discussed above) suggest that opioids may induce cell death. Indeed, in a study from our laboratory investigating the neurobiological effects of morphine following SCI, Hook et al. (2016) showed that acute treatment with this analgesic negatively impacts the survival of key cell types at the

lesion site. Subjects were administered incremental doses of intravenous morphine for 7 days following SCI, and tissue was collected 24 hours after the last dose for immunohistochemical analyses. The results showed that virtually no neurons or astrocytes remained across the rostral-caudal extent of the lesion in contused subjects treated with morphine (Hook et al., 2016). Furthermore, although not significant, these subjects also showed decreased expression of microglia/macrophages and increased expression of markers for neurodegeneration than their saline-treated counterparts.

Supporting our findings, other studies have also shown that morphine displays toxic properties. For example, morphine-induced death has been observed in a study of human neurons and microglia in vitro (Hu et al., 2002). Increased cell death has also been observed in the brains of rodent subjects undergoing chronic morphine administration (Atici et al., 2004; Bekheet et al., 2010; Emeterio et al., 2006; L.-W. Liu et al., 2013). Additionally, morphine also promotes monocyte and lymphocyte death (Bhat et al., 2004; Singhal et al., 1999; Singhal et al., 1998; Yin et al., 2006), an observation that may explain immune suppression in heroin addicts (Govitrapong et al., 1998). Although still under investigation, research suggests that opioids may induce cell death by promoting excitotoxicity, apoptosis, and inflammation.

#### *Excitotoxicity*

Evidence suggests that opioid-induced cell death may be a result of excitotoxicity. Prolonged opioid administration often results in the development of dependence, tolerance, and paradoxical pain (Bekhit, 2010; Ueda & Ueda, 2008). Opioid-induced alterations in N-methyl-D-aspartate receptor (NMDA receptor) signaling



have been strongly implicated in these effects (Mao et al., 1994; Marek et al., 1991; Trujillo & Akil, 1991). For example, co-administration of the noncompetitive NMDA receptor antagonist MK-801 attenuates the development of tolerance and dependence to chronic morphine, without affecting the analgesic efficacy of the drug (Mao et al., 1994; Marek et al., 1991; Trujillo & Akil, 1991). Repeated morphine administration has also been shown to increase protein kinase C (PKC) expression, activation, and translocation to the cell membrane (Mao et al., 1995; Mayer et al., 1995). Importantly, PKC can increase calcium influx by removing the magnesium blockade from the NMDA receptor/calcium channel (L. Chen & Huang, 1992). Furthermore, chronic morphine exposure is also associated with downregulation of glutamate transporters (Mao et al., 2002a; L. Yang et al., 2008) and enhanced excitatory neurotransmitter release from primary afferents (Gardell et al., 2002). Altogether, the evidence suggests that opioids directly and indirectly contribute to heightened excitation in the spinal cord, which may potentiate the central sensitization and excitotoxic cell death inherent to the injury process.

### *Apoptosis*

Opioid-induced cell death has been linked with increased expression of a variety of apoptosis-related proteins (Boronat et al., 2001; Emeterio et al., 2006; Hu et al., 2002; L.-W. Liu et al., 2013; Singhal et al., 1999; Willner et al., 2014; Yin et al., 1999; Yin et al., 2006). In vitro, morphine promotes apoptosis of neural progenitor cells, neurons, and microglia via a Caspase-3 mechanism, and this effect is blocked by naloxone (Hu et al., 2002; Willner et al., 2014). In vivo, addiction studies have found significantly increased

expression of pro-apoptotic Fas and Caspase-3, accompanied by decreased expression of anti-apoptotic Bcl-2, in the brains of rats undergoing prolonged morphine administration (L.-W. Liu et al., 2013). Similarly, chronic morphine-treated mice exhibit neuron and astrocyte death throughout the brain, as well as upregulated FasL, Fas, Bad, and the active fragments of caspases 3 and 8 (Emeterio et al., 2006). In Jurkat cells and human T lymphocytes, morphine-induced cell death appears to depend on the generation of reactive oxygen species, activation of the Fas/p53 pro-apoptotic pathway, and inhibition of the phosphatidylinositol 3-kinase (PI3K)/Akt anti-apoptotic pathway (Singhal et al., 1999; Yin et al., 2006). Decreased levels of the pro-survival phosphorylated protein kinase Akt, however, have also been shown in the brain under chronic morphine conditions (Muller & Unterwald, 2004).

### *Inflammation*

Indirectly, morphine may also induce cell death by activating immune-competent cells and increasing the release of pro-inflammatory cytokines. Opioid-induced glial activation has been associated with decreased analgesic efficacy, dependence, tolerance, and paradoxical pain (Hutchinson et al., 2007; Raghavendra et al., 2002; Raghavendra et al., 2004; Watkins et al., 2009). In vitro, lumbar dorsal spinal cord sections show significant increases in the release of IL-1 $\beta$ , IL-6, fractalkine, GRO/KC, MIP-1 $\alpha$ , MCP-1 and TNF- $\alpha$  following 180 minutes of incubation with 100  $\mu$ M morphine compared to media alone (Hutchinson et al., 2008a). In vivo, increases in IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and TNF- $\alpha$  have also been demonstrated after 7 days of intrathecal morphine (Hutchinson et al., 2008a). Importantly, pro-inflammatory cytokines can affect cell health and viability

(Buntinx et al., 2004; Hermann et al., 2001; Lee et al., 2000; van Kralingen et al., 2013; Ye et al., 2013). For example, astrocyte cultures treated with IL-1 $\beta$  and TNF $\alpha$  show compromised function (such as decreased cell adhesion and ATP production) as early as 24 hours post-treatment and significant cell death by 96 hours. Similarly, in oligodendrocyte cell lines cultured in the presence of TNF $\alpha$  and IFN- $\gamma$ , cell death is evidenced as early as 24 hours post-treatment in a dose dependent fashion (Buntinx et al., 2004).

#### *Synergistic effects of opioids and spinal cord injury*

Importantly, opioids may act synergistically with processes innate to SCI to increase cell death and attenuate recovery of function (Woller & Hook, 2013). The extent of damage during SCI is determined by the primary injury, and the secondary injury processes that immediately follow. It is during this secondary phase of injury that damage spreads from the epicenter of the lesion, to the rostral and caudal segments of the spinal cord. As opposed to the primary injury, medical intervention is possible during the secondary phase of injury to minimize the extent of the damage to surviving spinal tissue. On the other hand, opioid administration during this phase of SCI may exacerbate secondary injury, increasing cell death, and leading to decreased recovery of function. For example, following SCI, there is a significant increase in the release of glutamate and other excitatory amino acids (D. Liu et al., 1991). This contributes to increased NMDA, AMPA, and kainate receptor activity and the activation of voltage-gated ion channels (Dumont et al., 2001; Kwon et al., 2004). Subsequently, calcium and sodium influx increase to lethal levels, resulting in excitotoxicity. Combined with opioid-

induced increases in NMDA receptor activation and downregulation of glutamate transporters, this could significantly increase damage at the lesion site.

Furthermore, vascular disruption following SCI results in hemorrhage and ischemia (Mautes et al., 2000; Tator & Koyanagi, 1997). During ischemia, reactive oxygen species are generated, including superoxide, hydroxyl radicals, and nitric oxide, leading to oxidative stress (Jia et al., 2012). These toxic factors contribute to secondary damage during reperfusion of the spinal cord through oxidation of proteins, lipids, and nucleic acids. Morphine treatment also promotes the production of reactive oxygen species, such as nitric oxide (Chandel et al., 2012; Singhal et al., 1998). Reactive oxygen species can compromise key mitochondrial functions, resulting in metabolic distress and triggering apoptosis via cytochrome c release and caspase activation (Dumont et al., 2001; Kwon et al., 2004). As discussed above, prolonged morphine administration can also activate pro-apoptotic pathways in a variety of cells, decreasing tissue sparing at the injury site.

Lastly, inflammation is innate to SCI; however, an unchecked immune response can also increase collateral damage at the lesion site (Donnelly & Popovich, 2008; Fleming et al., 2006; Popovich et al., 1997). After SCI, the first response to the site of damage is by neutrophils, which secrete lytic enzymes and cytokines (Taoka et al., 1997). Then, circulating macrophages are recruited to the lesion site and resident microglia become activated (Popovich et al., 1997). The role of these phagocytes is crucial to the resolution of injury, since they are necessary of the clearance of dead cells and debris (Kigerl et al., 2009). On the other hand, the pro-inflammatory products

released by these cells also increase glial activation, chemotaxis, and cell proliferation. As previously mentioned, opioid administration also results in glial activation and the release of pro-inflammatory cytokines (Hutchinson et al., 2007; Hutchinson et al., 2008a; Hutchinson et al., 2011; Watkins et al., 2009). In the pathophysiological context of a SCI, opioids may trigger a positive feedback loop of pro-inflammatory cytokine production that can exacerbate the neurotoxic environment and result in further tissue damage. Paradoxically, opioid treatment following SCI may also result in aberrant glial activation, promoting the development of chronic pain (Christensen & Hulsebosch, 1997; Crown et al., 2006; Detloff et al., 2008; Gwak et al., 2012; Hulsebosch, 2008; Hutchinson et al., 2011; Watkins et al., 2009).

Overall, the extant literature suggests that opioids should not be used following SCI. As previously mentioned, however, simply abstaining from using these potent analgesics for the treatment of post-SCI pain is not an option. Instead, the mechanisms underlying morphine's adverse effects need to be elucidated to ensure that this drug can be safely used in the clinical setting. I propose that the beneficial (analgesia) versus adverse (undermined recovery) effects of opioids may be mediated by different opioid receptor subtypes. Next, I will discuss classic and non-classic opioid receptor signaling.

### **The opioid receptors**

#### *Classic opioid receptors*

The term "classic" refers to three major opioid receptor subtypes: the mu ( $\mu$  or MOR), delta ( $\delta$  or DOR), and kappa ( $\kappa$  or KOR) opioid receptors (Y. Chen et al., 1993; Evans et al., 1992; Kieffer et al., 1992; Meng et al., 1993). The classic opioid receptors

belong to the class A (Rhodopsin) group of receptors, and form part of the G-protein coupled receptor (GPCR) superfamily (HUGO Gene Nomenclature Committee, n.d.). In humans, these receptors are encoded in the *OPRM1*, *OPRD1*, and *OPRK1* genes, localized in chromosomes 6, 1, and 8, respectively (HUGO Gene Nomenclature Committee, n.d.). These genes are highly conserved; indeed, the classic opioid receptors are identical in approximately 60% of their amino acid sequence (Sobczak et al., 2014). The classic opioid receptors also share many structural similarities. These receptors are formed by seven transmembrane helical domains connected by three extracellular and three intracellular loops, with an extracellular N-terminus and an intracellular C-terminus (Filizola & Laakkonen, 1999; Law et al., 1999). While the transmembrane domains and intracellular loops are comparable across all three opioid receptor subtypes, the N-terminus, C-terminus, and extracellular loops can differ to varying degrees (Law & Loh, 2006). This divergence results in differences in ligand binding profiles and intracellular signaling.

In the absence of exogenous opioid compounds, opioid receptor activity occurs through binding of endogenous ligands produced under physiological conditions. Three main precursor proteins, pro-opiomelanocortin (POMC), proenkephalin, and prodynorphin, give rise to multiple active opioid peptides upon cleavage (Akil et al., 1984; Khachaturian et al., 1985). Their products- endomorphins, enkephalins, and dynorphins- show high affinity for the MOR, DOR, and KOR respectively. The major site of endogenous opioid production is the pituitary; however, opioid peptides have also been identified areas of the hypothalamus, the nucleus of the solitary tract, the nucleus

commissuralis, the spinal cord, and regions outside of the nervous system such as the adrenal medulla and along the gastrointestinal tract. Furthermore, immune cells also release opioid peptides, and play an important role in peripheral mechanisms of intrinsic opioid antinociception (Rittner et al., 2001; Schäfer et al., 1994; Stein et al., 1990). For example, in response to peripheral inflammation, opioid-containing immune cells can migrate to inflamed tissue and release opioid peptides (Schäfer et al., 1994). Stress can also trigger opioid release from immune cells by a mechanism involving corticotropin-releasing hormone (Schäfer et al., 1994).

Similar to the widespread production of endogenous opioid peptides, early autoradiography binding studies provided evidence that all three of the classic opioid receptors are widely distributed throughout the nervous system (Atweh & Kuhar, 1977a, 1977b, 1977c; Gouardères et al., 1985; Mansour et al., 1987; Tempel & Zukin, 1987). More recently, with the use of *in situ* hybridization and immunohistochemical techniques, localization of these receptors has been achieved with better resolution and higher specificity (Ding et al., 1996; Mansour et al., 1996; Mansour et al., 1995a; Mansour et al., 1995b; Mansour et al., 1994).

The differential expression of the classic opioid receptors across the nervous system has been linked with a variety of important physiological and psychological functions. For instance, opioid receptor mRNA has been localized to various nuclei of the hypothalamus and the pituitary, suggesting a role for opioid modulation of hormonal responses. In fact, altered endocrine function has been observed in patients receiving long-term treatment with opioids. These patients usually exhibit sex hormone

suppression, a condition also known as “opioid-induced hypogonadism”, which can result in severe erectile dysfunction and decreased libido, among other symptoms (Abs et al., 2000; Daniell, 2002; N. Katz & Mazer, 2009). Additionally, mRNA in the nucleus accumbens, ventral tegmental area (VTA), substantia nigra, and striatum underscore the role of opioid receptor activation in mechanisms of drug abuse and addiction (Lutz & Kieffer, 2013a). Localization to mesolimbic areas like the amygdala and hippocampus, coupled with modulation of dopamine centers, also supports a role of the opioid receptor systems in emotional processing and affect (Filliol et al., 2000; Kang et al., 2000; König et al., 1996; Lutz & Kieffer, 2013b; Ragnauth et al., 2001).

The localization of opioid receptors throughout the pain circuits of the nervous system is particularly important in the context of SCI. For instance, opioid receptor mRNA has been localized in dorsal root ganglia (DRG), the spinal cord, the spinal trigeminal nuclei, reticular nuclei, raphe nuclei, the periaqueductal gray (PAG), and the thalamus- areas involved in ascending and descending pain modulation (Mansour et al., 1995a; Mansour et al., 1994). At the supraspinal level, opioid receptors modulate pain by acting on neurons of the ventromedial medulla, which project to the spinal dorsal horn inhibiting pain transmission. This is an example of “descending” pain control. Opioid receptors also play an important role in the regulation of “ascending” pain signals. In primary sensory neurons, for example, opioid receptors are synthesized and transported to central and peripheral terminals. Centrally, they can act presynaptically to inhibit neurotransmitter release to the spinal cord. Peripherally, opioid receptors on cutaneous tissue can regulate pain and itch, and may also play a role in skin disorders and wound



healing (Bigliardi et al., 2009; Rook et al., 2008; Salemi et al., 2005; Taneda et al., 2011).

Aside from modulating the activity of these different neuronal populations throughout the PNS and CNS, opioid compounds can also act on a variety of other cell types that also express opioid receptors under normal physiological conditions. For example, opioid receptors have been detected on skin cells such as keratinocytes, fibroblasts, and epidermal melanocytes (Bigliardi-Qi et al., 2003; Cheng et al., 2008; Kauser et al., 2003). With the use of RT-PCR, opioid receptor expression has also been quantified in other human tissues, including lung, spleen, kidney, heart, skeletal muscle, liver, thymus, small intestine, pancreas, and adrenal gland (Peng et al., 2012). Further supporting an interaction between the immune and opioid systems, studies have demonstrated that cells involved in host defense, including circulating monocytes and peritoneal macrophages, also express opioid receptor mRNA (Bidlack et al., 2006; Chuang et al., 1995; Sharp, 2006). Even within the nervous system, opioid receptor expression is not restricted to neurons. Astrocytes, oligodendrocytes, and microglia also express opioid receptors (Chao et al., 1996; Chao et al., 1997; Ruzicka et al., 1995; Stiene-Martin et al., 1998; Tryoen-Toth et al., 2000).

Opioid receptor signal transduction mechanisms resulting in antinociception have been well characterized (Law, 2011; Law & Loh, 2006; Law et al., 2000). Like other GPCRs, opioid receptors signal through intracellular G (guanine nucleotide-binding) proteins. G proteins are heterotrimers composed of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -subunits. Following the binding of the ligand to the opioid receptor, GDP bound to the  $\alpha$ -subunit is released,

allowing the binding of GTP. This results in the dissociation of the G protein into two different signaling units:  $G\alpha$  and  $G\beta\gamma$ . These subunits then interact with other effectors and intracellular proteins to decrease neuronal activity. For instance,  $G\alpha$  subunits have been shown to inhibit adenylyl cyclase, resulting in decreased cAMP and PKA production.  $G\alpha$  subunits can also activate G protein-coupled inwardly rectifying potassium (KIR) channels at the postsynaptic membrane, hyperpolarizing the cell. Furthermore,  $G\beta\gamma$  subunits can inhibit pre- and postsynaptic voltage-gated calcium ( $Ca^{2+}$ ) channels, blocking  $Ca^{2+}$  influx into the cell, and reducing neurotransmitter release.

Aside from these well-known signal transduction pathways involved in the inhibitory effects of opioids, the classic opioid receptors may also play a role in other important signaling cascades. For example, activation of the classic opioid receptors has also been shown to modulate the MAP kinase pathways, composed of three protein kinase cascades: the extracellular-signal regulated kinases (Erks), the Jun *N*-terminal kinases (JNKs), and the p38 kinases (Law, 2011; Law & Loh, 2006; Law et al., 2000). Although not fully understood, potential mechanisms through which opioids activate these important signaling pathways are beginning to emerge. For example, selective agonists for the MOR and KOR can activate the ERK/MAPK cascade via secondary messengers like PKC (Belcheva et al., 2005). Additionally, intracellular proteins regularly recruited during opioid receptor activation, like the  $\beta$ -arrestins, may not only play a role in receptor trafficking and endocytosis, but may also activate MAPKs through the formation of complexes with other effector proteins (Bruchas et al., 2006). Overall, activation of MAPK signaling suggests that, aside from their well-known

analgesic effects, opioids and opioid receptors may also play a role in regulating numerous important cellular processes, including differentiation, survival, and apoptosis (Tegeder & Geisslinger, 2004).

#### *Non-classic opioid receptors*

One property of opioids that has facilitated the study of non-classic binding sites is stereoselectivity. The classic opioid receptors bind to conventional opioid drugs in a stereoselective fashion, meaning that they can bind to [-]-, but not [+]- isomers. These [+]- enantiomers, also known as unnatural opioids, have been widely used to investigate non-classic opioid binding in the nervous system. For example, in one of the earliest studies in opioid binding, Goldstein et al. (1971) used the morphine congener levorphanol and its enantiomer, dextrorphan, in the mouse brain. Among their most noteworthy results, Goldstein and colleagues showed that nonspecific binding sites are widely spread throughout the mouse brain. Furthermore, nonspecific sites greatly outnumbered stereospecific sites, constituting approximately 50% and 2% of overall binding respectively. Unfortunately, this non-stereospecific binding was not fully characterized in the original study, and was not pursued further in any subsequent work. Therefore, many questions remain as to the nature of these non-stereoselective sites. Recent findings, however, suggest these sites may represent opioids binding to non-classic receptors on glia, such as toll-like receptors (TLRs), resulting in immunomodulatory and anti-analgesic effects.

Many experiments published in recent years are helping to elucidate the role of the toll-like receptors (TLRs) in opioid-induced glial activation, decreased analgesic

efficacy, and the development and maintenance of pain (Hutchinson et al., 2011; Nicotra et al., 2012). The toll-like receptors are important components of the innate immune system (Kawasaki & Kawai, 2014). To date, over thirteen TLRs have been identified: TLR 1-10 in humans; TLR1-9, and TLR11-13 in mice. Together, they form part of a wide class of pattern recognition receptors (PRRs) that detect danger signals in the cellular environment. These signals include pathogen-associated microbial patterns (PAMPs) typically expressed by microbial pathogens and danger-associated molecular patterns (DAMPs) released from dying cells. Structurally, TLRs are type I transmembrane proteins, consisting of extracellular leucine-rich repeats (LRRs) and a cytoplasmic Toll/IL-1 receptor domain (Stirling et al., 2004). Upon recognition of PAMPs or DAMPs, individual TLRs can induce different signaling cascades by recruitment of adaptor proteins. Five of these proteins have been identified, including MyD88, Mal (MyD88 adaptor-like protein), TRIF (TIR domain-containing adaptor protein-inducing IFN- $\beta$ ), TRAM (TRIF-related adaptor molecule), and SARM (sterile  $\alpha$ - and armadillo-motif-containing protein). Signaling is achieved by coupling of the adaptor proteins to downstream kinases that activate a variety of transcription factors, such as NF- $\kappa$ B, ultimately leading to the production of pro-inflammatory cytokines and chemokines.

TLR4, the receptor that recognizes lipopolysaccharide (LPS), appears to play a particularly important role in opioid-glia interactions. For instance, research suggests that opioid ligands can bind to TLR4 and its accessory molecules in a non-stereoselective manner, activating glia, and initiating the inflammatory response

(Hutchinson et al., 2010a; Hutchinson et al., 2010b; Lewis et al., 2010; Wang et al., 2012). As mentioned previously, the release of pro-inflammatory substances can undermine opioid analgesia and potentiate pain. These effects, however, can be blocked with LPS-specific antagonists like LPS-RS, and non-stereoselectively by the use of unnatural opioid antagonists like [+] - naloxone (Hutchinson et al., 2008b; Hutchinson et al., 2010b). Genetic studies further support the role of TLR4 in opioid-induced inflammation. TLR4 knockout mice show a three-fold increase in morphine analgesia when compared to their wildtype counterparts (Hutchinson et al., 2010b). Importantly, in a model of sciatic nerve chronic constriction injury, non-stereoselective inhibition of TLR4 by intrathecal [+] - naloxone and [+] - naltrexone suppressed microglial activation and successfully reversed symptoms of neuropathic pain (Hutchinson et al., 2008b). These findings suggest that TLR4 could be targeted pharmacologically to improve the safety and efficacy of clinically-employed opioid analgesics.

### **Specific aims**

Morphine can bind stereoselectively to the classic opioid receptors (MOR, DOR, and KOR), but it can also activate non-classic receptors in a non-stereoselective fashion (Kristensen et al., 1994; Wang et al., 2012). Despite the traditional categorization of morphine as a selective MOR agonist, research shows that morphine also binds to the DOR and KOR, although with approximately 70 and 40 less affinity than MOR (Kristensen et al., 1994). Therefore, morphine may exert its adverse effects through any of the opioid-receptor systems, alone or in combination. The aim of the experiments reported in Chapters 3-5 (outlined in Figure 1) was to identify the critical receptor

subtypes involved in the morphine-induced attenuation of locomotor recovery after SCI. Based on the extant literature, three primary mechanisms were identified that may result in morphine-induced attenuation of function following SCI.

First, repeated MOR activation may contribute to neurotoxicity and the development of paradoxical pain observed with morphine administration. Whereas activation of the MOR is typically associated with analgesia, Mao et al. (1995) proposed that repeated activation of this receptor system leads to a PKC-mediated decoupling of the receptor from its associated G-protein and decreased morphine-induced antinociception. Binding of morphine to the MOR is also thought to initiate G-protein mediated protein kinase C (PKC) translocation and activation, promoting the removal of the NMDA receptor  $Mg^{+}$  plug (L. Chen & Huang, 1992) and allowing  $Ca^{2+}$  influx. An increase in intracellular  $Ca^{2+}$  leads to activation of additional PKC, production of nitric oxide (NO), and regulation of gene expression. NO may act as a retrograde messenger to enhance glutamate release from the presynaptic neuron, which would further potentiate activity in the postsynaptic neuron. Mao and colleagues have also shown that chronic morphine exposure is associated with downregulation of glutamate transporters, directly contributing to the heightened activity of NMDA receptors (Mao et al., 2002a; L. Yang et al., 2008). Overall, sustained potentiation of the NMDA receptor maintains central sensitization and hyperalgesia in the neural system, and may lead to excitotoxic cell death (Mao et al., 2002b; Woolf & Thompson, 1991).

Morphine may also undermine recovery of function after SCI by activating the KOR system. Behaviorally, Faden and colleagues have demonstrated that intrathecal

administration of dynorphin (an endogenous KOR ligand) causes hindlimb paralysis in neurologically intact rats, and blocking the KOR improves neurological outcomes after a contusion SCI (Faden & Jacobs, 1983; Faden et al., 1987). KOR activation may also increase glial activation, leading to decreased recovery of function. For example, Xu and colleagues (2007) explored the cellular consequences of KOR activation in a model of partial sciatic nerve ligation. This injury normally results in increased astrocyte proliferation in the spinal dorsal horn. However, the same manipulation failed to show astrocyte proliferation in dynorphin knock-out mice, mice with a homozygous KOR deletion, or mice treated with the specific KOR antagonist norBNI. Importantly, in the same study, blocking downstream KOR targets *in vivo* for 7 days following partial sciatic nerve ligation resulted in reduced spinal astrocyte proliferation, an effect that correlated with decreased signs of neuropathic pain, such as allodynia and hyperalgesia.

Morphine may also exert its negative effects through activation of non-classic opioid receptors that play an important role in immunity (Hutchinson et al., 2007; 2008b; Tawfik et al., 2005; Watkins et al., 2009). For example, research suggests that opioid ligands can bind to TLR4 and its accessory molecules in a non-stereoselective manner, leading to glial activation and initiating the inflammatory response (Hutchinson et al., 2010a; Hutchinson et al., 2010b; Lewis et al., 2010; Wang et al., 2012). The subsequent release of pro-inflammatory cytokines in the spinal cord has been shown to oppose opioid analgesia and facilitate pain (Hutchinson et al., 2008a; Johnston et al., 2004). In the SCI model, we have also shown that morphine administration results in increased pro-inflammatory cytokine expression (IL-1 $\beta$  and IL-6) at the injury site, and that

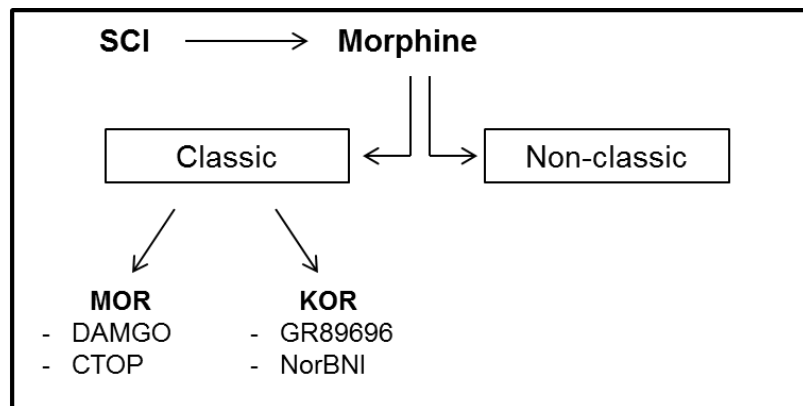
blocking the IL-1 receptor during morphine administration prevents morphine-induced attenuated recovery (Hook et al., 2011). These data suggest that non-classic opioid receptor signaling may be key mechanism contributing to the morphine-induced attenuation of function.

To address these hypotheses, in the first set of experiments (Chapter 3), I evaluated the contribution of MOR and KOR activation to the morphine-induced attenuation of function observed following experimental SCI using selective opioid receptor agonists and antagonists. In the second set of experiments (Chapter 4), I assessed the contribution of TRL4 using a selective agonist and antagonist. I also assessed overall non-classic signaling and glial activation using [+] morphine and minocycline, respectively. In the last set of experiments (Chapter 5), I assessed the effects of morphine at the lesion site by quantifying key cell populations using immunohistochemistry. Since I posited that opioid-immune interactions may significantly contribute to morphine's adverse effects, I also investigated the immune response and classic opioid receptor co-localization on microglia and macrophages using flow cytometry. The aim of the experiments reported here was to further our understanding of opioid receptor signaling within the pathophysiological context (spinal cord injury) in which they are prescribed. This avenue of research has been largely overlooked, but merits further investigation in order to improve the safety and efficacy of current pain medications for the spinally-injured population.



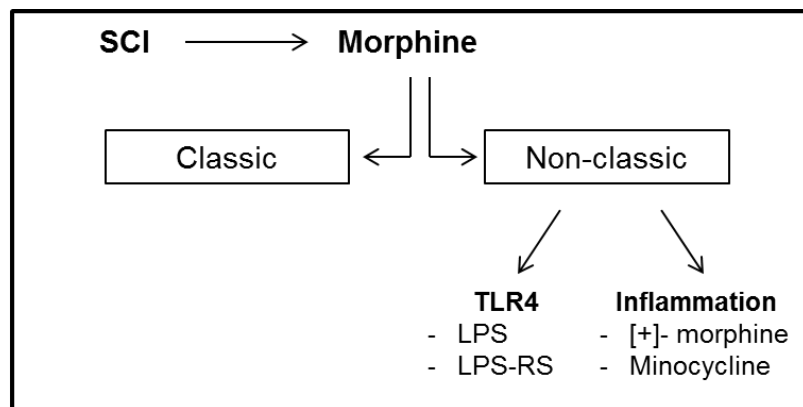
### Aim 1: Classic opioid receptors

The aim of these experiments was to evaluate the contribution of the classic opioid receptors to the morphine-induced attenuation of function following SCI.



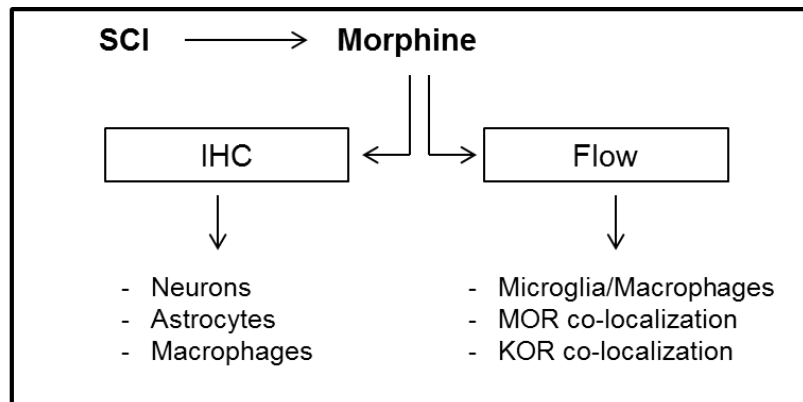
### Aim 2: Non-classic opioid receptors

The aim of these experiments was to evaluate the contribution of the non-classic opioid receptors to the morphine-induced attenuation of function following SCI.



### Aim 3: Opioid-immune interactions

The aim of these experiments was to assess the effects of morphine treatment on cellular survival and the immune response at the lesion site.



*Figure 1: Specific aims.* This is an outline of the experiments presented here. Aims 1, 2, and 3 correspond to Chapters 3, 4, and 5 respectively.

## CHAPTER II

### GENERAL METHODOLOGY

#### **Subjects**

The subjects were male Sprague-Dawley rats obtained from Harlan (Houston, TX, USA). They were approximately 90-110 days old (300-350 g) and were individually housed in Plexiglas bins [45.7 (length) x 23.5 (width) x 20.3 (height) cm] with food and water continuously available. Following surgery, subjects were manually expressed in the morning (7:00-8:30 a.m.) and in the evening (4:30-6:00 p.m.) until they regained full bladder control (operationally defined as three consecutive days with an empty bladder at the time of expression). Subjects were checked daily for signs of autophagia and spastic hypertonia. A subject was classified as having spastic hypertonia if the limb was in an extended, fixed position and was resistant to movement. The subjects were maintained on a 12 hour light/dark cycle and all behavioral testing was conducted during the light cycle.

All of the experiments reported here were reviewed and approved by the Institutional Animal Care Committee at Texas A&M University and all NIH guidelines for the care and use of animal subjects were followed.

#### **Surgery**

Subjects received a moderate contusion injury using the Infinite Horizon spinal cord impactor (PSI, Fairfax Station, VA, USA). Briefly, subjects were anesthetized with isoflurane (5%, gas), and after a stable level of anesthesia was reached, the concentration

of isoflurane was lowered to 2-3%. The subject's back was shaved and disinfected with iodine and a 5.0 cm incision was made over the spinal cord. Two incisions were made along the vertebral column, on each side of the dorsal spinous processes, extending about 2 cm rostral and caudal to the T12 segment. Muscle and connective tissue were then dissected to expose the underlying vertebral segments. Musculature around the transverse processes was cleared to allow for clamping of the vertebral spinal column. Next, the dorsal spinous process at T12 was removed (laminectomy), and the spinal tissue exposed (approximately L1-L3). The dura remained intact. The vertebral column was fixed within the IH device using two pairs of Adson forceps. A moderate injury was produced using an impact force of 150 kdynes and a 1 s dwell time.

After injury, a 15-cm-long polyethylene (PE-10) cannula, fitted with a stainless steel guiding wire (P01008, Ernie Ball Inc., Coachella, CA, USA), was threaded 2 cm under the vertebrae immediately caudal to the injury site. The tubing was inserted into the subarachnoid space. To prevent cannula movement, the exposed end of the tubing was secured to the vertebrae rostral to the injury using tissue adhesive (3M Vetbond Tissue Adhesive, 3M Animal Care Products, St Paul, MN, USA). The wire was then pulled from the tubing and the wound was closed using Michel clips. To help prevent infection, subjects were treated with 100 000 units/kg Pfizerpen (penicillin G potassium) immediately after surgery and again 2 days later. For the first 24 h after surgery, rats were placed in a recovery room maintained at 26.6 °C. To compensate for fluid loss, subjects were given 3 ml of saline after surgery. Michel clips were removed 14 d after surgery.

## Drug administration

Drug administration took place on the day after surgery, following baseline tests of locomotion and thermal reactivity. Baseline BBB scores were balanced across all groups. Drugs were administered via an intrathecal route. The following drugs were used: [-]- Morphine (NIDA Drug Supply Program), DAMGO ([D-Ala<sup>2</sup>, NMe-Phe<sup>4</sup>, Gly-ol<sup>5</sup>]-enkephalin; Tocris Bioscience, Bristol, UK), CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH<sub>2</sub>; Tocris Bioscience, Bristol, UK), GR89696 (4-[(3,4-Dichlorophenyl)acetyl]-3-(1-pyrrolidinylmethyl)-1-piperazinecarboxylic acid methyl ester fumarate salt; Sigma-Aldrich, St. Louis, MO, USA), NorBNI (nor-Binaltorphimine; Tocris Bioscience, Bristol, UK), LPS (Lipopolysaccharide from *Escherichia coli*; Sigma-Aldrich, St. Louis, MO, USA), LPS-RS Lipopolysaccharide from *Rhodobacter sphaeroides*; InvivoGen, San Diego, CA, USA), [+]- morphine (NIDA Drug Supply Program), minocycline (minocycline hydrochloride; Sigma-Aldrich, St. Louis, MO, USA). Filtered saline (0.9%) was used to flush catheters following the injection of a drug at a volume of 10 µL.

## Assessment of sensory reactivity

### *Thermal reactivity*

To assess acute drug efficacy, the tail-flick test (D'amour & Smith, 1941; Jeffrey et al., 2001; Šedý et al., 2008) of thermal reactivity was used immediately before and after treatment. Subjects were placed in restraining tubes and allowed to acclimate to the tail-flick apparatus (IITC Life Science Inc., Woodland Hills, CA, USA) and testing room (maintained at 26.5°C) for 15 min. Prior to testing, the temperature of the light, focused

on the tail, was set to elicit a baseline tail-flick response in approximately 4 s in an intact rat. This pre-set temperature was then maintained across the SCI subjects. In testing, the latency to flick the tail away from the radiant heat source (light) was recorded. If a subject failed to respond, the test trial was automatically terminated after 8 s of heat exposure. Two tests occurred at 2-minute intervals, and the last tail-flick latency was recorded.

#### *Tactile reactivity*

To evaluate the long-term recovery of sensory function, reactivity thresholds were also assessed after day 21 post-injury. Thermal reactivity was evaluated using the tail-flick test, as described above. Mechanical reactivity was tested by applying von Frey filaments (Semmes-Weinstein Anesthesiometer, Stoelting Co., Chicago, IL, USA) to the plantar surface of the hindpaws, in a manner previously described by others (Chaplan et al., 1994; Jeffrey et al., 2001; Šedý et al., 2008). Briefly, stimuli were presented every 2 seconds until rats exhibited a motor (hindpaw withdrawal) and vocal response. The intensity of the stimuli that produced a response was reported using the formula provided by Semmes-Weinstein:  $\text{Intensity} = \log_{10}(10,000 * \text{g force})$ . If one or both responses (motor and vocal) were not observed, testing was terminated at a force of 300 g. Each rat was tested twice on each hindpaw in a counterbalanced ABBA order (A= left paw, B= right paw).

#### *Girdle reactivity*

Tactile reactivity was also assessed at the level of injury using the girdle test (Christensen & Hulsebosch, 1997). To ensure that the rats remained calm for testing,

they were handled for 5 minutes immediately prior to beginning the girdle test. For this test, the girdle region was shaved and a von Frey filament with bending force of 204.14 mN (26 g force) was applied across a grid covering the girdle region. The filament was applied to 44 points (4 across the back of the subject and 11 down), and vocalization responses were recorded and mapped onto a grid map of that animal. Since animals do not normally vocalize to this stimulus, a vocalization response indicated that a noxious stimulus was experienced. In mapping the area of response, the number of vocalizations are recorded ( $N_v$ ) and normalized by the following formula:  $(N_v \times 100)/\text{total number of applications (44)}$ , indicating the percent vocalizations out of the total number of applications.

### **Assessment of locomotor recovery**

#### *BBB scoring*

Locomotor behavior was assessed for 21 days post-injury, using the Basso, Beattie and Bresnahan (BBB) scale (Basso et al., 1995; Šedý et al., 2008) in an open enclosure (a blue children's wading pool, 99 cm in diameter, 23 cm deep). Baseline motor function was assessed on the day following injury and prior to drug treatment. Locomotor behavior was then scored once per day for 1 week (days 2–7). Subjects were scored every other day from day 9 to day 15 and every third day on days 18 and 21. Because rodents often remain motionless (freeze) when first introduced to a new apparatus, subjects were acclimated to the observation fields for 5 min per day for 3 days prior to surgery. Each subject was placed in the open field and observed for 4 min. Care was taken to ensure that all investigators' scoring behavior had high intra- and inter-

observer reliability (all  $r$ 's > 0.89) and that they were blind to the subject's experimental treatment.

### *Tapered beam and ladder walk tests*

Additional measures of motor recovery were obtained at the end of the 21-day recovery period using tapered beam and ladder walk tasks (Šedý et al., 2008). Prior to testing, subjects were habituated to the experimental context for 3 days (8 min per day). During this period of familiarization, they were trained to traverse a wide beam (48.3 cm) and enter a black box positioned at the end of the beam runway. The beginning of the runway was brightly lit, motivating subjects to move toward the dark box. They were left in the box for 2 min after they had traversed the beam. Subjects were then tested on the tapered beam and ladder.

The beam walk test (Schallert, 2002; Schallert & Woodlee, 2005) provides a comparative index of the postural stability of the rats, as well as a gross measure of paw placement abilities. The ledged, tapered beam was 6.75 in (17.15 cm) wide at the start, and 0.375 in (0.95 cm) wide at the end. The width at which each hindpaw failed to plantar place on the beam was recorded. The average width across the two legs was used as an index of beam walk performance.

The ladder task (Metz & Whishaw, 2002; Šedý et al., 2008) provides a measure of the extent to which experimental manipulations affect the fine motor abilities of the hindpaws. The rats were required to cross a horizontal ladder (20 cm wide; 37 rungs at 2.5 cm spacing) in order to reach the black box. The number of times that the rats did not successfully place their hindpaws (their paws slipped between the rungs) was assessed

using post hoc frame-by-frame video analyses. Each hindpaw was scored according to the number of errors made, with lower scores indicating better ladder walk performance (a maximum of 11 errors was recorded if no plantar placement was observed per hindpaw).

### **Histology**

At the end of behavioral testing, subjects were deeply anesthetized (100 mg/kg of beuthanasia, i.p.) and perfused intracardially with 4% paraformaldehyde. A 1-cm-long segment of the spinal cord, that included the lesion center, was taken and prepared for cryostat sectioning. The tissue was sectioned coronally (20  $\mu\text{m}$ ) and every 10<sup>th</sup> slice was preserved for staining. All sections were stained with cresyl violet for Nissl substance and luxol fast blue for myelin (Beattie, 1992; Behrmann et al., 1992).

The total cross-sectional area of the cord and spared tissue was assessed at the lesion center using Neurolucida software (MFB Bioscience, Williston, VT, USA). Sections  $\pm$  600, 1200, 1800, and 2400  $\mu\text{m}$  from the lesion center (rostral and caudal) were also traced and analyzed. Four indices of lesion magnitude were derived: lesion, residual gray matter, residual white matter, and width. To determine the area of lesion, an observer who was blind to the experimental treatments, traced around the boundaries of cystic formations and areas of dense gliosis (Basso et al., 1995). Nissl-stained areas that contained neurons and glia of approximately normal densities denoted residual gray matter. White matter was judged spared in myelin-stained areas lacking dense gliosis and swollen fibers. The total area of each cross-section was derived by summing the areas of damage, and gray and white matter. Width was determined from the most lateral



points across the transverse plane. These analyses yielded six parameters for each section: white matter area, gray matter area, spared tissue (white + gray), damaged tissue area, net area (white + gray + damage), and section width.

To control for variability in section area across subjects, a correction factor was applied derived from standard undamaged cord sections taken from age-matched controls. This correction factor is based on section widths and is multiplied by all area measurements to standardize area across analyses (Grau et al., 2004). By standardizing area across sections, it is possible to estimate the degree to which tissue is ‘missing’ (i.e., tissue loss from atrophy, necrosis, or apoptosis). An accurate assessment of the degree to which a treatment has impacted, or lesioned, the cord includes both the remaining damaged tissue as well as resolved lesioned areas. By adding the amount of missing tissue and the measured damaged area, an index of the relative lesion (% relative lesion) can be derived in each section that is comparable across sections. Similarly, this allows an estimation of the relative percent of gray and white matter remaining in each section, relative to intact controls. These measures are highly correlated with various measures of behavioral performance including BBB locomotor scores and recovery of bladder function (Grau et al., 2004).

### **Immunohistochemistry**

For immunohistochemical analyses, spinal cord sections mounted on Superfrost Plus slides were washed ( $3 \times 10$  min) in phosphate-buffered saline (1X PBS), then incubated in blocking solution (3% normal goat serum, 0.1% Triton X-100 in 1X PBS) for 1 hour at room temperature. A total of three sets of slides were used (see Table 1)

Primary antibodies were diluted in blocking solution, applied to the slides, and allowed to incubate overnight at room temperature on an orbital at gentle speed. The following day, all slides were washed ( $3 \times 10$  min) in cold 1X PBS, and incubated in the appropriate Alexa fluor-conjugated secondary antibodies prepared in blocking solution for 2 hours at room temperature. After another series of washes in 1X PBS ( $3 \times 10$  min), the slides were mounted in Prolong Gold + DAPI antifading mounting medium (Life Technologies, NY, USA) and coverslipped.

To quantify immunofluorescence, images of spinal sections  $\pm 600, 1200, 1800,$  and  $2400 \mu\text{m}$  from the lesion center (rostral and caudal) were captured using a Virtual Tissue 2D system in Stereo Investigator (MFB Bioscience, Williston, VT) at 4X magnification. The Virtual Tissue 2D program aligns, stitches, and blends the section together into a montage, providing a complete image of the total spinal section. Digital image analyses were performed using ImageJ Software (U. S. National Institutes of Health, Bethesda, Maryland, USA). Basic cell counting in ImageJ was used to quantify neurons. Fluorescence thresholds were set and background was subtracted from all images. The size and circularity of the target particles were defined and the counts generated by the program were recorded. Astrocyte and microglia/macrophage fluorescence was quantified using densitometric features in ImageJ. First, the perimeter of each section was digitally outlined. A threshold value was obtained for each image ensuring that all labeled cells were selected (i.e., target area). The magnitude of fluorescent expression was reported as integrated density, obtained by multiplying the “Integrated Density” by “Counts” generated by the program.

Table 1. List of antibodies used for immunohistochemistry.

| Primary        | Manufacturer          | Dilution | Secondary       | Manufacturer          | Dilution | Set |
|----------------|-----------------------|----------|-----------------|-----------------------|----------|-----|
| NeuN (chicken) | Abcam (Cambridge, UK) | 1:500    | Alexa Fluor 633 | Abcam (Cambridge, UK) | 1:500    | 1   |
| GFAP (chicken) | Abcam (Cambridge, UK) | 1:500    | Alexa Fluor 633 | Abcam (Cambridge, UK) | 1:500    | 2   |
| OX-42 (mouse)  | Abcam (Cambridge, UK) | 1:500    | Alexa Fluor 633 | Abcam (Cambridge, UK) | 1:500    | 3   |

Table 2. List of antibodies used for flow cytometry.

| Target | Antibody                      | Manufacturer                            | Conj. Dye       | Dilution** | Plate |
|--------|-------------------------------|---|-----------------|------------|-------|
| CD11b  | Anti-CD11b                    | Abcam (Cambridge, UK)                   | Phycoerythrin   | 1:100      | 1, 2  |
|        | Anti-Rat CD11b/c              | eBioscience (San Diego, CA, USA)        | eFluor 660      | 1:100      | 3     |
| IBA1   | Anti-Iba1                     | Abcam (Cambridge, UK)                   | Alexa Fluor 647 | 1:100      | 1, 2  |
|        | Anti-Iba1                     | Abcam (Cambridge, UK)                   | FITC            | 1:200      | 3     |
| CD45   | Anti-Rat CD45                 | eBioscience (San Diego, CA, USA)        | eFluor 450      | 3:100      | 1, 3  |
| CD86   | Biotin anti-rat CD86          | BioLegend (San Diego, CA, USA)          | *               | 2:100      | 1     |
| CD68   | Anti-CD68                     | LifeSpan Biosciences (Seattle, WA, USA) | FITC            | 9:400      | 1     |
| CD206  | Macrophage Mannose Receptor 1 | Antibodies-Online (Atlanta, GA, USA)    | *               | 2:100      | 2     |
| MOR    | Anti- $\mu$ -Opioid Receptor  | Alomone Labs (Jerusalem, Israel)        | ATTO-488        | 1:100      | 2     |
| KOR    | Anti-kappa Opioid Receptor    | Abcore (Ramona, CA, USA)                | Cy3             | 1:100      | 3     |

\* Biotinylated antibody conjugated to PE/Cy7 Streptavidin (BioLegend, San Diego, CA, USA)

\*\* Per 150,000 cells in 200  $\mu$ L of solution

### **Flow cytometry**

Subjects were deeply anesthetized (100 mg/kg of beuthanasia, i.p.) and perfused intracardially with 100 ml of cold 1X PBS. A 1-cm-long segment of the spinal cord including the lesion center was collected, mechanically dissociated using a blade, washed in X-VIVO 15 (Lonza, Basel, Switzerland), and retrieved by centrifugation (300 X G, 5 min, 4 degrees Celsius). The tissue was then enzymatically (Neural Dissociation Kit P, Miltenyi Biotec, Bergisch Gladbach, Germany) and mechanically dissociated. The cell suspension was washed in X-VIVO 15 and pelleted by centrifugation (300 X G, 10 min, 4 degrees Celsius). The cell pellet was resuspended in 6 ml of X-VIVO 15, and applied to an Opti-Prep gradient. The gradient was composed of four 1-ml layers of Opti-prep diluted in X-VIVO 15, arranged in the following order (from bottom to top): 35%, 25%, 20%, and 15%. The 10 ml tube containing the cell suspension and gradient was centrifuged at 726 X G for 15 minutes at 20 degrees Celsius with low acceleration and no brake. Following centrifugation, the top 7 ml of solution, containing myelin and debris, was aspirated and discarded. The remaining 3 ml, containing a heterogeneous mixture of cells (including inflammatory cells, neurons, astrocytes, etc.), were filtered through a 30  $\mu$ m strainer, washed twice in X-VIVO 15, and counted (Countess, Life Technologies, Carlsbad, CA, USA). The single-cell suspension was then pipetted into three 96-well plates and incubated with antibodies for the following markers: CD11b, IBA1, CD45, CD86, CD68, CD206, MOR, and KOR (see Table 2).

For each subject, 2 technical replicates were prepared and phenotyped using a FACSFortessa flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with

FlowJo software (FlowJo, LLC, Ashland, OR, USA). 100,000 events were read per sample. The following gating strategy was used to identify cells of interest. “Live” cells were selected using forward and side scatter, which enabled the removal of dead cells and debris. To control for cellular autofluorescence, unstained samples were prepared. To be considered positive (+), cell populations were selected using a gate that contained <1% unstained cells. Microglia/macrophages were identified by selecting cells that were positive for both, CD11b and IBA1 markers. To differentiate between infiltrating macrophages and resident microglia, a CD45 marker was used. CD45+ cells were selected and further separated into discrete populations of high and low based on fluorescence intensity. For all further analysis (i.e. CD86/CD86, MOR/CD206, KOR/CD206), a quadrant system was created to determine phenotype and receptor expression.

### **Statistical analysis**

The results were analyzed using analysis of variance (ANOVA). When main effects were found to be significant, the Bonferroni test was used for post-hoc analyses. Planned comparisons were also included in our assessment of the data when more than one drug treatment was used. These were performed in anticipation of dose-dependent effects of adjuvants on morphine’s efficacy, even in the absence of overall main effects. This also allowed for independent comparisons between morphine and vehicle-treated groups, regardless of the adjuvant or dose used. Lastly, in experiments with a continuous independent variable (e.g., recovery of locomotor function across days and histology), mixed-design ANOVAs were used.

Locomotor scores using the BBB scale were transformed, as previously described by our lab (Ferguson et al., 2004) to help assure that the data were amenable to parametric analyses. Briefly, this transformation removes a discontinuity in the scale, which justifies the use of parametric statistical analyses, and increases statistical power. Additional statistical power was also achieved by obtaining a measure of locomotor performance 24 h after injury, prior to drug treatment. Using day 1 as a covariate in an analysis of covariance (ANCOVA) substantially reduces unexplained variance and thereby increases statistical power.

## CHAPTER III

### EVALUATION OF THE CLASSIC OPIOID RECEPTORS\*

#### Introduction

As previously discussed in Chapter I, despite its clinical utility, morphine administered following SCI has been shown to produce a variety of unwanted side effects, including reduced functional recovery and increased paradoxical pain (Hook et al., 2007; Hook et al., 2009; Hook et al., 2011; Woller et al., 2014; Woller et al., 2012). These findings are alarming and suggest that morphine may be contraindicated in the days following trauma. However, without a viable alternative to opioids, simply abstaining from use of these potent analgesics is unfeasible. Instead, elucidating the mechanisms underlying the adverse effects of morphine and similar medications is imperative in order to ensure their safety and efficacy in the clinical setting.

Investigation of the molecular pathways underlying the effects of opioids must begin with the primary targets of these drugs- the classic opioid receptors. The  $\mu$  (mu)-opioid receptor (MOR),  $\delta$  (delta)-opioid receptor (DOR), and  $\kappa$  (kappa)-opioid receptor (KOR) are the key binding sites for endogenous and exogenous opioids, and are collectively known as the “classic opioid receptors.” Opioids differentially engage these

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\* Part of the data reported in this chapter is reprinted from Aceves, M., Mathai, B.B., & Hook, M.A. (2016). Evaluation of the effects of specific opioid receptor agonists in a rodent model of spinal cord injury. *Spinal Cord*, 54(10), 767-777 (Copyright 2016 by the authors) and Aceves, M., Bancroft, E.A., Aceves, A.R., & Hook, M.A. (2017). Nor-Binaltorphimine blocks the adverse effects of morphine after spinal cord injury. *Journal of Neurotrauma*, 34(6), 1164-1174 (Copyright 2017 by Mary Ann Liebert, Inc.).

receptors, resulting in varied positive and negative effects. Although the mechanism(s) of action of morphine and other opioids at these classic receptors has been well described, there is a lack of research looking at the effects of these drugs in the pathophysiological context of an injury. Indeed, the research outlined below suggests that the adverse effects of morphine following SCI may depend on classic opioid receptor signaling.

Morphine binds to the MOR with high affinity, but also binds with lower affinity to the DOR and KOR (Kristensen et al., 1994). Morphine may exert its adverse effects through any of these receptor systems. For example, whereas activation of the MOR is typically associated with analgesia, binding of morphine to the MOR is also thought to initiate G-protein mediated protein kinase C (PKC) translocation and activation, promoting the removal of the NMDA receptor  $Mg^{+}$  plug and allowing  $Ca^{2+}$  influx (L. Chen & Huang, 1992; L. Chen & Marine, 1991). Chronic morphine exposure is also associated with down regulation of glutamate transporters, directly contributing to the heightened activity of NMDA receptors (Mao et al., 2002a; L. Yang et al., 2008). In this way, morphine may act through the MOR to potentiate NMDA receptor activation, maintaining central sensitization and hyperalgesia in the neural system, and leading to excitotoxic cell death (Mao et al., 2002b; Woolf & Thompson, 1991).

The KOR system has also been implicated in detrimental effects following trauma. For example, Faden and colleagues showed that dynorphin, the endogenous ligand of the KOR, and dynorphin-related peptides induce paraplegia even when administered to the intact spinal cord (Faden & Jacobs, 1983; Herman & Goldstein,



1985). Prodynorphin mRNA expression and dynorphin immunoreactivity are also significantly elevated in the spinal cord following trauma, and correlate with injury severity and neurological dysfunction (Cox et al., 1985; Faden et al., 1985a; Faden et al., 1985b; Przewłocki et al., 1988; Tachibana et al., 1998). Importantly, administration of intrathecal dynorphin exacerbates functional deficits after SCI, whereas treatment with dynorphin antiserum improves outcome after trauma (Faden, 1990). These data strongly suggest that the adverse effects of morphine following SCI may be mediated by the KOR system.

Based on the extant literature, the following experiments evaluated the contribution of MOR and KOR activation to the morphine-induced attenuation of function observed following experimental SCI (due to a lack of evidence supporting a detrimental role of DOR activation, this receptor system was not assessed). In order to more clearly dissociate the beneficial (analgesic) and deleterious consequences of morphine treatment, these opioid receptor systems were probed using selective opioid receptor agonists and antagonists. The data presented here support a role for the KOR, but not the MOR, in the adverse effects of morphine administration following SCI. The results indicate increased involvement of the spinal KOR system in attenuated functional recovery, increased paradoxical pain, and decreased tissue sparing at the lesion site following injury.

## Methods and results

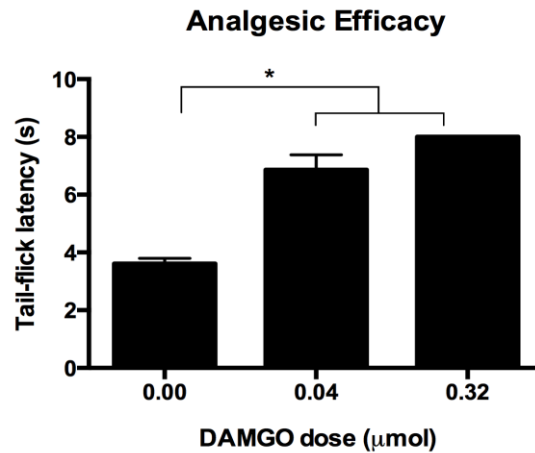
### *The $\mu$ -opioid receptor system*

#### **Exp. 1A Sufficiency of MOR activation**

In this experiment I used the highly selective MOR agonist DAMGO to assess the contribution of the MOR system to the morphine-induced attenuation of function after SCI. To test whether selective activation of MORs is sufficient to reproduce the adverse effects of morphine following spinal injury, DAMGO was dissolved in distilled water (filtered 0.9% saline was used as the vehicle control) and administered via an intrathecal route, as described in Chapter II General Methodology. Three DAMGO doses were tested (0, 0.04, and 0.32  $\mu$ mol). With an n=8 per group, this experimental design used a total of 24 rats.

#### *Analgesic efficacy of DAMGO*

Sensory function was evaluated, using the tail-flick test, to establish baseline thermal reactivity thresholds. Prior to treatment, tail-flick scores ( $\pm$  S.E.M.) ranged from  $4.43 \pm 0.26$  s to  $4.73 \pm 0.52$  s. There were no significant differences between the groups at this time point ( $F(2, 21) = 0.10$ ,  $p > 0.05$ ; data not shown). An ANOVA on post-treatment scores, however, revealed a significant main effect of DAMGO on tail-flick latency ( $F(2, 21) = 44.30$ ,  $p < 0.05$ ). As shown in Figure 2, both groups receiving DAMGO had increased tail-flick latencies relative to vehicle-treated controls. At the highest dose (0.32  $\mu$ mol), subjects displayed maximal tail-flick latencies (the test was automatically terminated at 8 seconds to prevent tissue injury).



*Figure 2: Analgesic efficacy of DAMGO.* The results of the tail-flick test of thermal reactivity are depicted. DAMGO administration significantly increased tail-flick latencies relative to vehicle-treated controls. Results are shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests.

#### *Effects of DAMGO on locomotor recovery*

As can be seen in Figure 3, administration of DAMGO did not significantly affect recovery of locomotor function at any of the doses tested. Mean converted BBB scores ( $\pm$  S.E.M.) on day 1 ranged from  $2.13 \pm 0.36$  to  $2.25 \pm 0.39$ . Statistical analyses showed that locomotor scores did not differ across groups prior to drug treatment ( $F(2, 21) = 0.03$ ,  $p > 0.05$ ). A mixed-design ANOVA also showed that there were no significant group differences across the recovery period ( $F(2, 21) = 1.17$ ,  $p > 0.05$ ). Motor recovery was further evaluated at the end of the 21-day recovery period using the tapered beam and ladder walk tests (Figure 4). Treatment with DAMGO did not have a significant effect on either beam ( $F(2, 21) = 0.60$ ,  $p > 0.05$ ) or ladder performance ( $F(2, 21) = 1.56$ ,  $p > 0.05$ ).

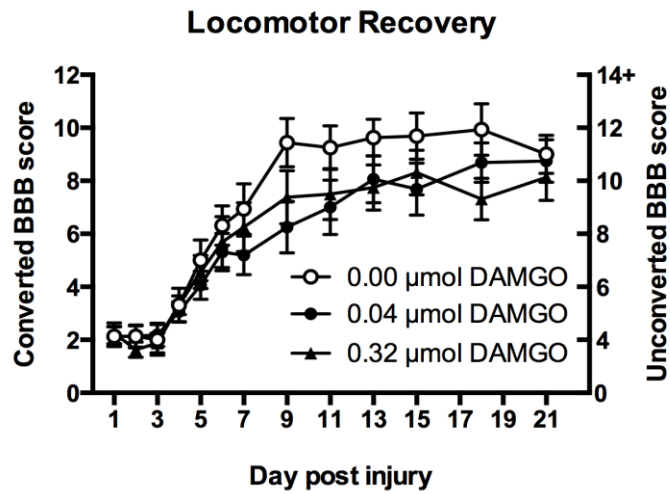


Figure 3: Effects of DAMGO on locomotor recovery. Subjects were monitored for 21 days following a moderate contusion SCI. Intrathecal administration of DAMGO, on day 1 post-injury, did not affect locomotor recovery. Results shown as Mean  $\pm$  S.E.M.

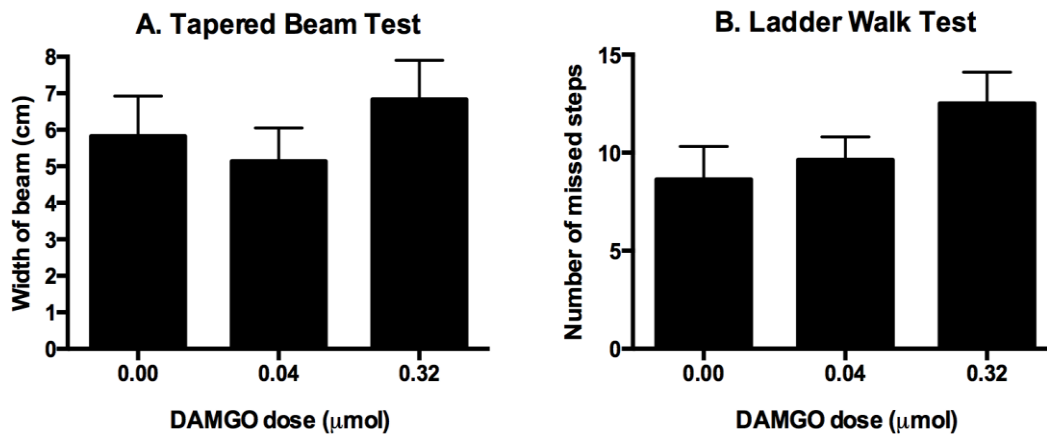
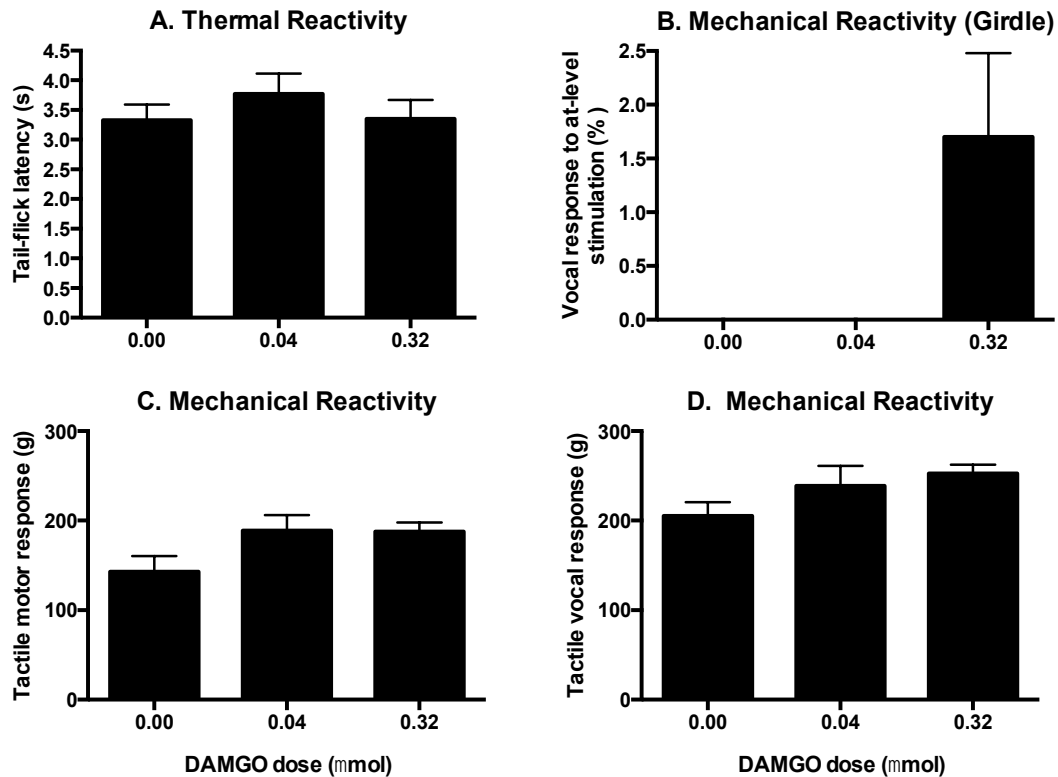


Figure 4: Effects of DAMGO on beam and ladder walk performance. At the end of the 21-day recovery period, locomotor recovery was further assessed using the tapered beam (A) and ladder walk (B) tests. DAMGO had no effect on the performance of these tests. Results shown as Mean  $\pm$  S.E.M.



*Figure 5: Effects of DAMGO on sensory reactivity.* At the end of the 21-day recovery period, sensory reactivity was assessed using the tail-flick (A), girdle (B), and von Frey (C, D) tests. A single dose of DAMGO did not affect long-term pain reactivity. Results shown as Mean  $\pm$  S.E.M.

#### *Effects of DAMGO on sensory reactivity*

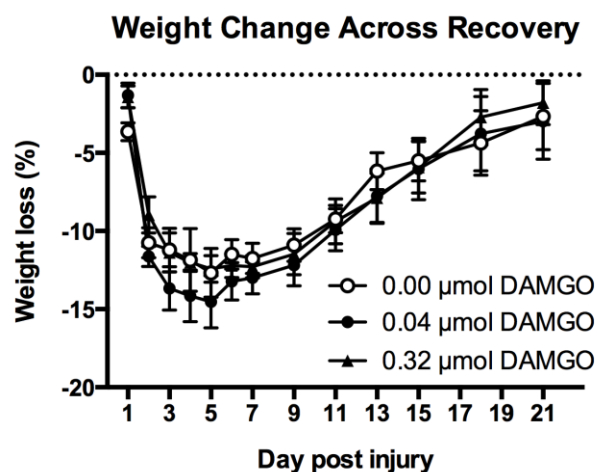
Sensory function was assessed at the end of the 21-day recovery period using the tail-flick, von Frey, and girdle tests (Figure 5). DAMGO did not have a significant effect on thermal reactivity ( $F(2, 21) = 0.57, p > 0.05$ ) assessed with the tail-flick test. Furthermore, there were no effects of DAMGO on motor ( $F(2, 21) = 2.51, p > 0.05$ ) or vocal reactivity ( $F(2, 21) = 1.87, p > 0.05$ ) to a mechanical stimulus applied to the hindpaws. An ANOVA, however, revealed a main effect of drug dose on the girdle test

( $F(2, 21) = 4.20, p < 0.05$ ), with vehicle controls showing more reactivity to at-level stimulation than either the 0.04  $\mu\text{mol}$  or 0.32  $\mu\text{mol}$  groups. This difference, however, was based on a comparison of 1.7% vocalizations by the vehicle controls, to zero vocalizations by the 0.04 and 0.32  $\mu\text{mol}$  groups, and thus may not be functionally significant.

#### *Effects of DAMGO on general health*

Weight was monitored as an index of general health. Immediately before injury, weight did not differ across groups ( $F(2, 21) = 2.49, p > 0.05$ ; data not shown). To assess changes in weight across recovery, the daily % weight loss was calculated by subtracting the starting weight (weight at the day of surgery) from daily weight. A repeated-measures ANOVA on daily % weight loss values showed that there was no significant effect of DAMGO ( $F(2, 21) = 0.30, p > 0.05$ ). As shown in Figure 6, all groups lost weight following SCI, and slowly regained it throughout the recovery period.

In addition to weight, mortalities, autophagia, spastic hypertonia, and recovery of bladder control were also recorded to assess general health. There were no mortalities or cases of spastic hypertonia observed in any of the treatment groups. Only one subject (treated with 0.32  $\mu\text{mol}$  DAMGO) showed signs of autophagia, and only one completely recovered bladder function (treated with vehicle).



*Figure 6: Effects of DAMGO on weight change across recovery.* This graph depicts the daily % weight loss across the 21-day recovery period, calculated by subtracting starting weight from daily weight. DAMGO did not affect weight loss, or other signs of general health, following SCI. Results shown as Mean  $\pm$  S.E.M.

### Exp. 1B Necessity of MOR activation

In this experiment I used the highly selective MOR antagonist CTOP to assess the necessity of MOR activation in the morphine-induced attenuation of function after SCI. To test whether inactivation of MORs blocks the adverse effects of morphine following SCI, subjects were pretreated with intrathecal CTOP (0 or 10  $\mu$ g dissolved in distilled water). One hour later, half of the subjects in each dose group were treated with 90  $\mu$ g of morphine and the remaining subjects were treated with vehicle (0.09% saline). The 1 hr waiting period between CTOP and morphine administration was chosen based on previous studies that demonstrate that CTOP binding in rat brain tissue reaches a steady state 30 to 90 minutes post-administration (Hawkins et al., 1989). This 2 X 2 experimental design (4 groups) used a total of 40 rats (n=10).

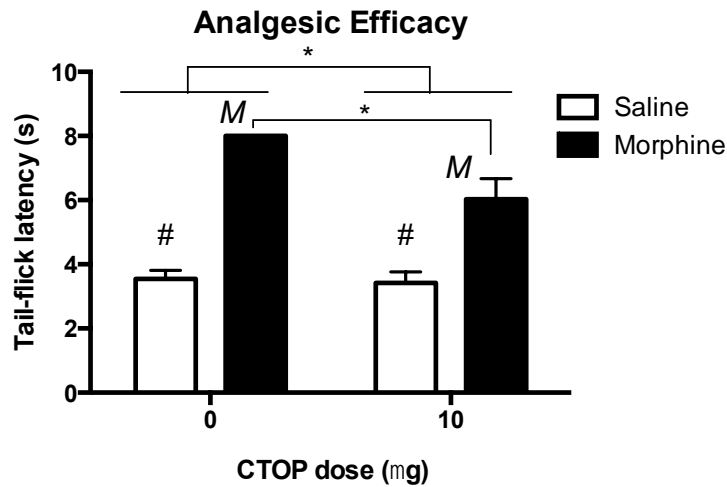
### *Effects of CTOP on the analgesic efficacy of morphine*

Sensory function was evaluated using the tail-flick test to establish baseline thermal reactivity thresholds. Prior to treatment, tail-flick scores ( $\pm$  S.E.M.) ranged from  $3.06 \pm 0.31$  s to  $4.00 \pm 0.34$  s. Statistical analyses showed that there were no significant differences between the groups at this time point ( $F(3, 35) = 2.16, p > 0.05$ ; data not shown). Post-treatment, a two-factor ANOVA revealed significant main effects of morphine ( $F(1, 35) = 73.36, p < 0.05$ ) and CTOP treatment ( $F(1, 35) = 6.51, p < 0.05$ ), as well as a significant morphine X CTOP interaction ( $F(1, 35) = 4.97, p < 0.05$ ) on tail-flick latency. As shown in Figure 7, both groups receiving morphine showed significantly increased tail-flick latencies compared to saline controls. Pre-treatment with  $10 \mu\text{g}$  CTOP significantly decreased the analgesic effects of morphine ( $p < 0.05$ ).

### *Effects of CTOP on locomotor recovery*

In addition to the effects on analgesia, I wanted to assess whether MOR activation was *necessary* to induce the morphine-induced attenuation of locomotor recovery. To address this, CTOP was administered as an adjuvant to morphine treatment 24 hours following SCI, and locomotor recovery was monitored for a 21-day period. Locomotor scores collected before treatment on day 1 did not differ significantly across groups ( $F(3, 36) = 0.32, p > 0.05$ ). Mean converted BBB scores ( $\pm$  S.E.M.) on day 1 ranged from  $1.75 \pm 0.26$  to  $2.20 \pm 0.49$ .





*Figure 7: Effects of CTOP on the analgesic efficacy of morphine.* The analgesic efficacy of morphine challenged with CTOP pre-treatment is depicted for the test of thermal reactivity. At both doses (0 and 10  $\mu$ g), subjects that received morphine had significantly increased tail-flick latencies relative to saline controls. However, morphine's analgesic effects decreased with CTOP pre-treatment. Results shown as Mean  $\pm$  S.E.M. \*  $p < 0.05$  for post-hoc tests; #  $p < 0.05$  for planned comparisons; M = significant main effect of morphine.

Converted BBB scores across recovery were analyzed using a mixed-design ANCOVA (with day 1 scores as a covariate). There was a significant main effect of morphine treatment on locomotor recovery ( $F(1, 35) = 12.71, p < 0.05$ ), but no effect of CTOP ( $F(1, 35) = 0.31, p > 0.05$ ) and no significant interaction between morphine and CTOP ( $F(1, 35) = 1.13, p > 0.05$ ). As shown in Figure 8, subjects that received morphine had lower converted BBB scores throughout recovery irrespective of CTOP treatment. Planned comparisons confirmed that control subjects (0  $\mu$ g CTOP) treated with morphine showed significantly reduced locomotor recovery when compared to their saline-treated counterparts ( $p < 0.05$ ), replicating our previous studies (Hook et al., 2009; Hook et al., 2011). At the 10  $\mu$ g dose, the difference between morphine and saline

treatment only approached significance ( $p = 0.07$ ). The 10  $\mu\text{g}$  CTOP + morphine group, however, showed significantly lower locomotor scores across recovery when compared to the control subjects not treated with CTOP ( $p < 0.05$ ).

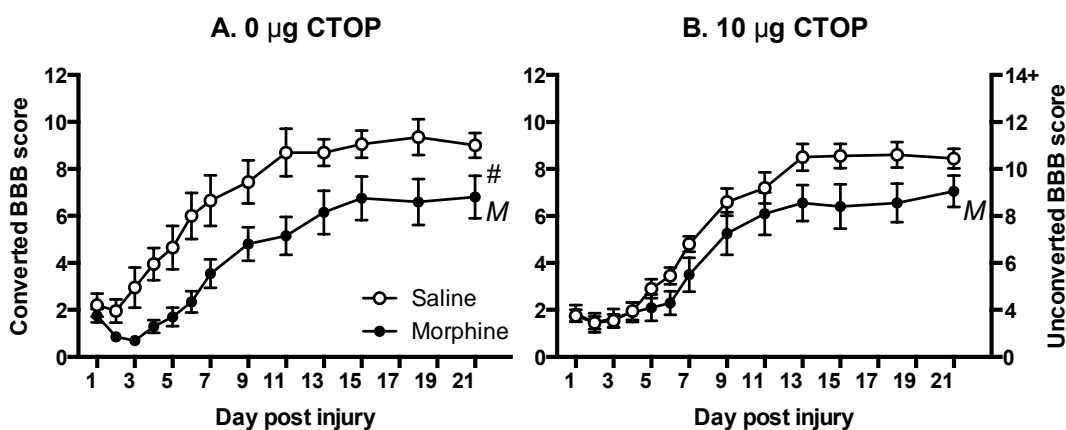
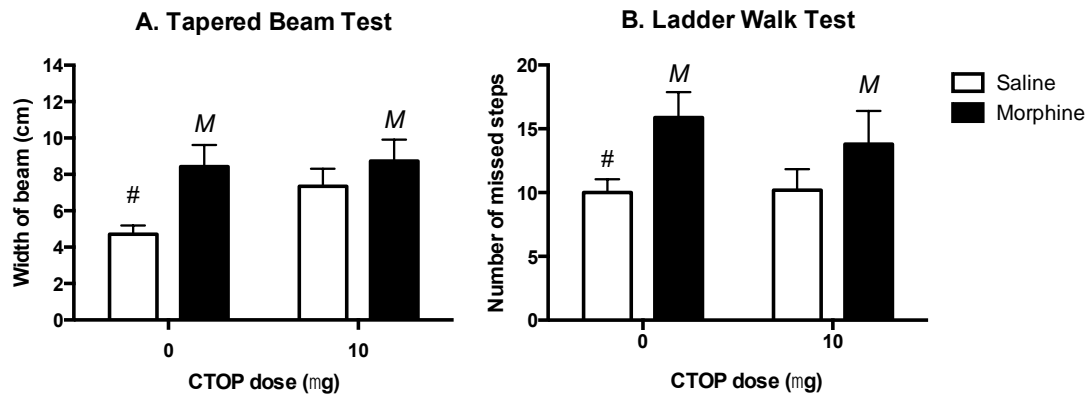


Figure 8: Effects of CTOP on locomotor recovery. Morphine significantly undermined locomotor recovery following SCI across doses. Planned comparisons revealed significant differences between morphine and saline-treated subjects at the 0  $\mu\text{g}$  dose of CTOP (A), but this effect did not reach significance with the 10  $\mu\text{g}$  dose (B). Results shown as Mean  $\pm$  S.E.M. #  $p < 0.05$  for planned comparisons; M = significant main effect of morphine.

Motor recovery was further evaluated at the end of the 21-day recovery period using the tapered beam and ladder walk tests (Figure 9). Commensurate with the BBB assessments, I found a significant main effect of morphine on beam performance ( $F(1, 36) = 5.88, p < 0.05$ ), but no effect of CTOP ( $F(1, 36) = 1.94, p > 0.05$ ) and no morphine X CTOP interaction ( $F(1, 36) = 1.25, p > 0.05$ ). Interestingly, on this task, saline-treated subjects at the 10  $\mu\text{g}$  dose of CTOP performed almost as poorly as morphine-treated animals. In the ladder walk test, morphine also significantly increased

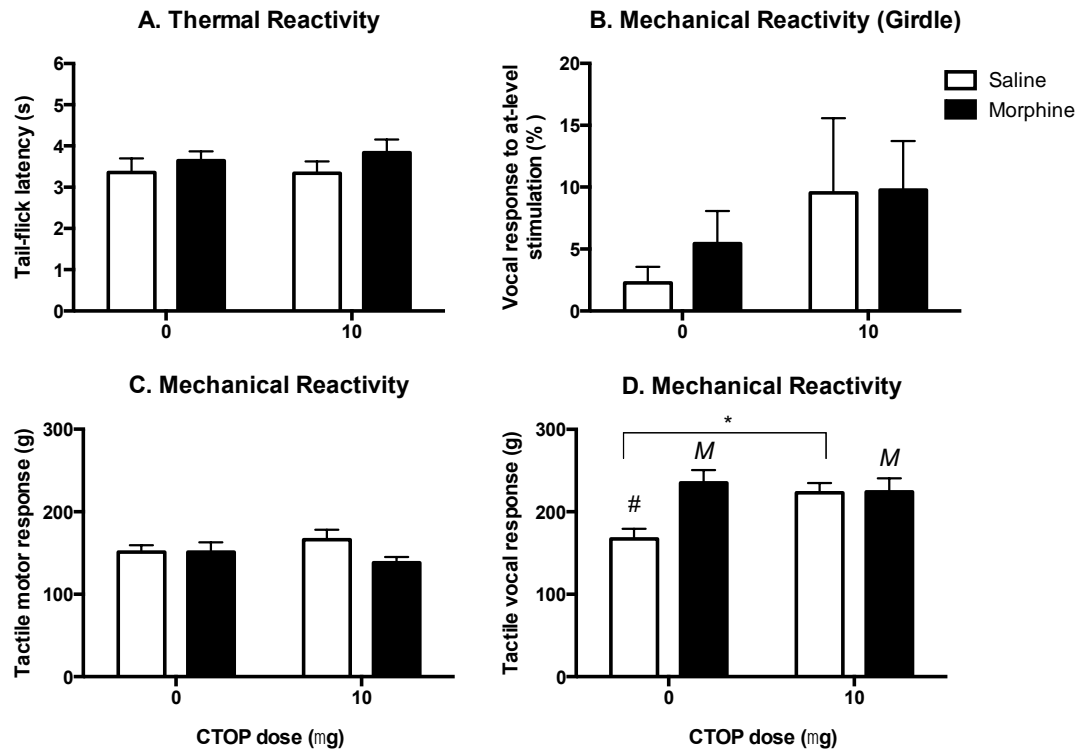
the number of possible errors, indicating decreased locomotor function ( $F(1, 36) = 5.59$ ,  $p < 0.05$ ). As in the beam test, CTOP did not have a significant effect ( $F(1, 36) = 0.22$ ,  $p > 0.05$ ) and no interaction between the drug treatments was observed ( $F(1, 36) = 0.33$ ,  $p > 0.05$ ).



*Figure 9: Effects of CTOP on beam and ladder walk performance.* At the end of the 21-day recovery period, locomotor recovery was further assessed using the tapered beam (A) and ladder walk (B) tests. Morphine significantly undermined performance in both tests. There was no effect of CTOP on the performance of either task. Results shown as Mean  $\pm$  S.E.M. #  $p < 0.05$  for planned comparisons; M = significant main effect of morphine.

#### *Effects of CTOP on sensory reactivity*

Long-term effects on sensory function were assessed at the end of the 21-day recovery period using the tail-flick, von Frey, and girdle tests (Figure 10). There was no effect of morphine ( $F(1, 36) = 1.54$ ,  $p > 0.05$ ), CTOP ( $F(1, 36) = 0.09$ ,  $p > 0.05$ ), or a morphine CTOP interaction ( $F(1, 36) = 0.13$ ,  $p > 0.05$ ) on the test of thermal reactivity.



**Figure 10: Effects of CTOP on sensory reactivity.** Drug treatment had no effect on the development of thermal hyperalgesia (A) or at-level allodynia (B). Mechanical reactivity of the hindpaws appeared to be affected by drug treatment, although this effect was only observed in vocal (D), but not motor (C), responses. Results shown as Mean  $\pm$  S.E.M. \* $p$  < 0.05 for post-hoc tests; #  $p$  < 0.05 for planned comparisons;  $M$  = significant main effect of morphine.

Similarly, I did not find any significant differences across the groups in our assessment of motor responses to von Frey stimulation. Analysis showed that there were no effects of morphine ( $F(1, 36) = 1.75, p > 0.05$ ) or CTOP ( $F(1, 36) = 0.01, p > 0.05$ ) treatment, and no interaction ( $F(1, 36) = 1.75, p > 0.05$ ). On the other hand, drug treatment appeared to affect vocal responses to tactile stimulation of the hindpaws. A two-factor ANOVA on vocal responses revealed a significant main effect of morphine

treatment ( $F(1, 36) = 5.27, p < 0.05$ ) and a significant interaction between morphine and CTOP ( $F(1, 36) = 5.00, p < 0.05$ ), but no main effect of CTOP administration ( $F(1, 36) = 2.24, p > 0.05$ ). Unexpectedly, as shown in Figure 10, saline controls showed lower vocal thresholds in comparison to the other three treatment groups at 21 days post injury and treatment.

Lastly, in the girdle test of at-level allodynia, I found no effect of morphine ( $F(1, 36) = 1.75, p > 0.05$ ) or CTOP ( $F(1, 36) = 0.01, p > 0.05$ ), and no interaction effect with the two drug treatments ( $F(1, 36) = 1.75, p > 0.05$ ).

#### *Effects of CTOP on general health*

Weight immediately before injury did not differ across groups ( $F(3, 36) = 0.57, p > 0.05$ ; data not shown). However, a repeated-measures ANOVA on daily % weight loss values revealed a significant main effect of morphine treatment ( $F(1, 36) = 7.41, p < 0.05$ ) and a significant morphine X CTOP interaction ( $F(1, 36) = 5.20, p < 0.05$ ), with no effect of CTOP alone ( $F(1, 36) = 0.80, p > 0.05$ ). As shown in Figure 11, morphine-treated subjects lost more weight than their saline-treated counterparts. Interestingly, CTOP administration appeared to augment weight loss in saline-treated animals, which lost significantly more weight across recovery than saline-treated animals not pre-treated with CTOP ( $p < 0.05$ ).

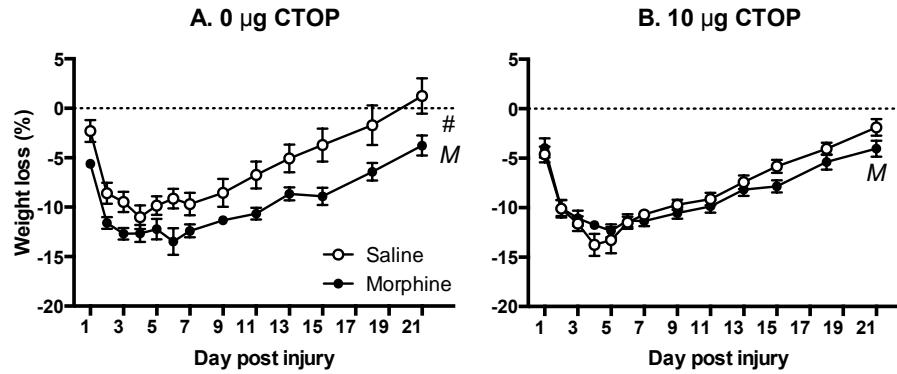


Figure 11: Effects of CTOP on weight change across recovery. The average daily % weight loss is depicted across the 21-day recovery period. Morphine significantly decreased weight across recovery. Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests; #  $p < 0.05$  for planned comparisons; M = significant main effect of morphine.

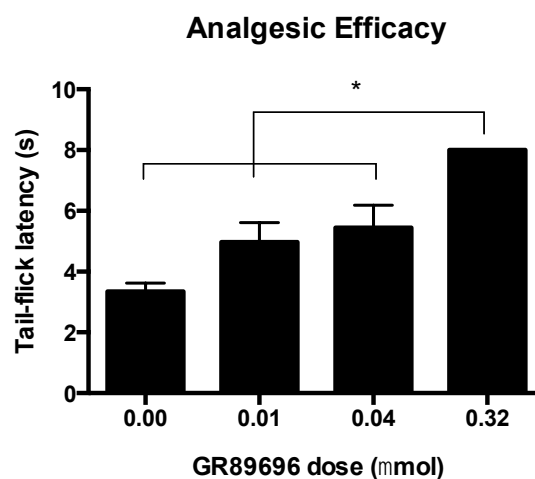
In addition to weight, mortalities, autophagia, spastic hypertonia, and recovery of bladder control were also recorded to assess general health. There were no mortalities and no cases of autophagia. Furthermore, only one subject (in the 0 µg CTOP + morphine group) showed signs of spastic hypertonia. Recovery of bladder control was observed in 55% of our subjects, as early as 7 days post-injury. To assess the effect of drug treatment on bladder function, I compared the latency to recovery (days) across conditions. A two-factor ANOVA showed that there were no effects of morphine ( $F(1, 36) = 0.02, p > 0.05$ ), CTOP ( $F(1, 36) = 1.54, p > 0.05$ ), or an interaction ( $F(1, 36) = 0.38, p > 0.05$ ) on the duration of neurogenic bladder symptoms.

## **Exp. 2A Sufficiency of KOR activation**

In this experiment I used the highly selective KOR agonist GR89696 to assess the contribution of the KOR system to the morphine-induced attenuation of function after SCI. To test whether selective activation of KORs in the spinal cord following injury is sufficient to attenuate recovery, GR89696 was dissolved in 34% DMSO solution (34% DMSO solution was also used as the vehicle control) and administered via an intrathecal route, as described in Chapter II General Methodology. Four doses of GR89696 were tested (0, 0.01, 0.04, and 0.32  $\mu\text{mol}$ ). With an  $n=8$  per group, this experimental design used a total of 32 rats.

### *Analgesic efficacy of GR89696*

Sensory function was evaluated using the tail-flick test to establish baseline thermal reactivity thresholds. Prior to treatment on day 1, tail-flick scores ( $\pm$  S.E.M.) ranged from  $3.56 \pm 0.27$  s to  $4.58 \pm 0.17$  s. Statistical analyses showed that there were no significant differences between the groups at this time point ( $F(3, 28) = 2.83$ ,  $p > 0.05$ ; data not shown). An ANOVA on post-treatment scores, however, revealed a significant main effect of GR89696 on tail-flick latency ( $F(3, 28) = 12.60$ ,  $p < 0.05$ ). As shown in Figure 12, groups receiving GR89696 had increased tail-flick latencies relative to vehicle-treated controls. There was a dose-dependent effect of GR89696 on the tail-flick response; at the highest dose of GR89696 (0.32  $\mu\text{mol}$ ) subjects displayed maximal latencies (8 seconds), an effect commensurate with morphine administration.



*Figure 12: Analgesic efficacy of GR89696.* The results of the tail-flick test of thermal reactivity are depicted. GR89696 administration significantly increased tail-flick latencies relative to vehicle-treated controls. Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests.

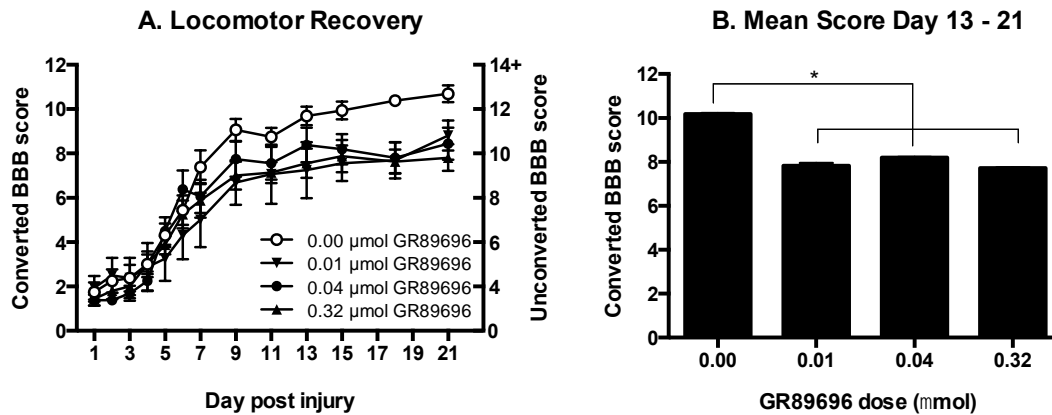
#### *Effects of GR89696 on locomotor recovery*

In contrast to DAMGO, administration of GR89696 in the acute phase of SCI undermined recovery of locomotor function at all doses tested (Figure 13). Locomotor scores collected before treatment on day 1 did not differ across groups ( $F(2, 21) = 0.40$ ,  $p > 0.05$ ). Mean converted BBB scores ( $\pm$  S.E.M.) ranged from  $1.38 \pm 0.25$  to  $1.94 \pm 0.53$ . There was no main effect of drug treatment ( $F(3, 27) = 2.00$ ,  $p > 0.05$ ) on locomotor recovery. However, using day 1 scores as a covariate, an ANCOVA revealed that there was a significant interaction between drug dose and BBB scores across the 21 day recovery period ( $F(33, 297) = 1.86$ ,  $p < 0.05$ ). An ANOVA comparing locomotor function from Days 13-21, when locomotor performances had stabilized, revealed a main effect of dose on locomotor function ( $F(3, 28) = 3.01$ ,  $p < 0.05$ ). As can be seen in

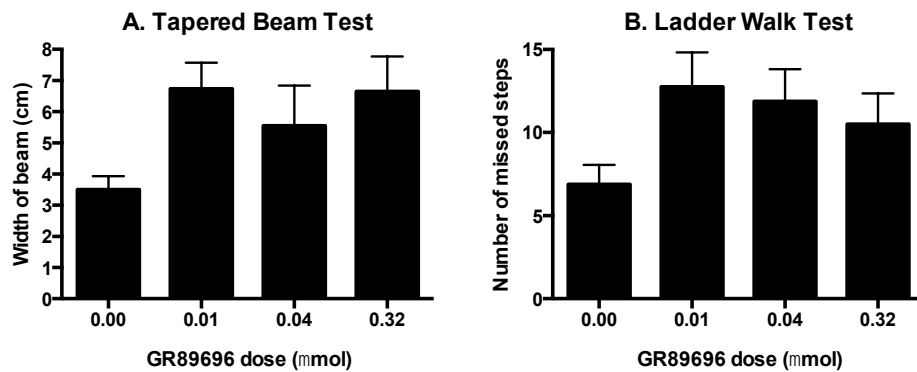


Figure 13, subjects treated with GR89696, irrespective of dose, displayed significantly lower levels of locomotor recovery relative to vehicle-treated controls ( $p < 0.05$ ).

Vehicle-treated controls also performed better than GR89696-treated subjects on additional tests of motor recovery. As shown in Figure 14, vehicle-treated controls walked across the narrow edge of the tapered beam with more success than experimental subjects and made fewer errors when traversing a ladder. These differences, however, did not reach statistical significance on either the beam ( $F(3, 28) = 2.10, p > 0.05$ ) or ladder walk tests ( $F(3, 28) = 1.83, p > 0.05$ ).



*Figure 13: Effects of GR89696 on locomotor recovery.* Subjects were assessed for 21 days following a moderate contusion SCI. Intrathecal administration of GR89696, on day 1 post-injury, undermined recovery of locomotor function at all doses tested (A). Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests.



*Figure 14: Effects of GR89696 on beam and ladder walk performance.* At the end of the 21-day recovery period, motor recovery was further assessed using the tapered beam (A) and ladder walk (B) tests. Although administration of GR89696 appeared to worsen performance in these tasks, the effect did not reach statistical significance ( $p < 0.05$ ). Results shown as Mean  $\pm$  S.E.M.

#### *Effects of GR89696 on sensory reactivity*

At 21 days post injury and treatment, there was no effect of GR89696 on thermal reactivity ( $F(3, 28) = 2.77, p > 0.05$ ), motor reactivity to tactile stimulation ( $F(3, 28) = 2.49, p > 0.05$ ) or at-level allodynia assessed with the girdle test ( $F(3, 28) = 0.29, p > 0.05$ ). However, as shown in Figure 15, ANOVAs uncovered significant differences for vocal reactivity to tactile stimulation of the hindpaws across doses ( $F(3, 28) = 2.95, p < 0.05$ ), with subjects in the 0.01  $\mu\text{mol}$  group showing decreased reactivity thresholds when compared with subjects in the 0.32  $\mu\text{mol}$  and control groups.

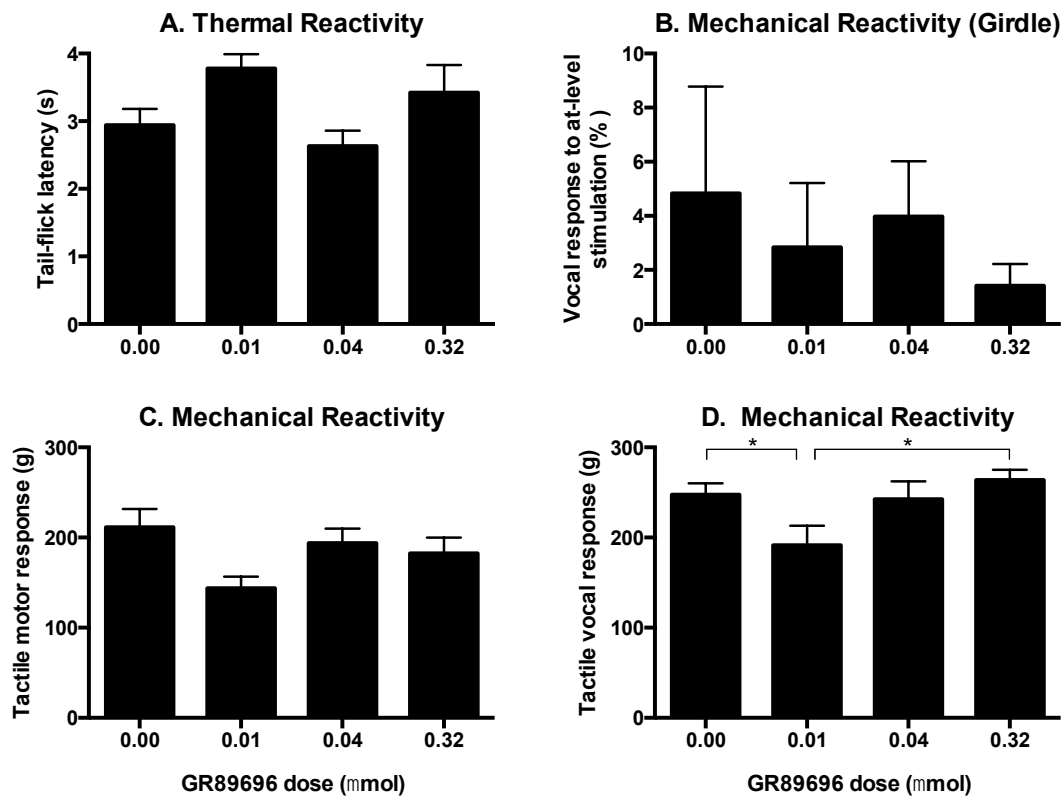
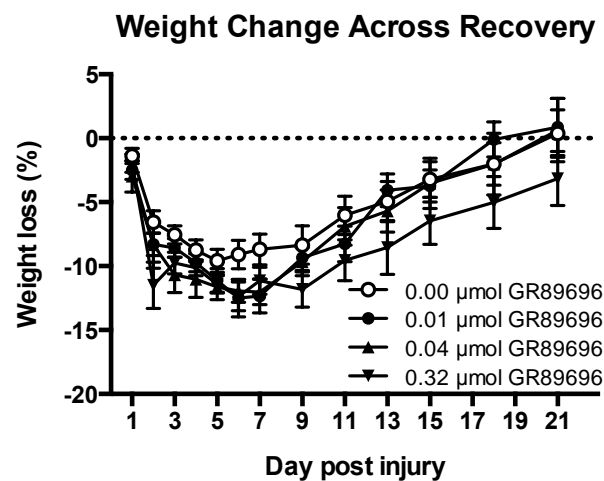


Figure 15: Effects of GR89696 on sensory reactivity. At the end of the 21-day recovery period, no effect of GR89696 on sensory reactivity was observed using the tail-flick (A), girdle (B), and von Frey test for motor responses (C). However, vocal responses to tactile stimulation were affected by dose (D). Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests.

#### Effects of GR89696 on general health

As in previous experiments, weight was monitored as an index of general health. Weight immediately before injury did not differ across groups ( $F(3, 28) = 0.32$ ,  $p > 0.05$ ; data not shown). All subjects showed decreased weight early after injury, which was slowly regained over time (Figure 16). A repeated-measures ANOVA on daily % weight loss values revealed no significant effect of GR89696 treatment on weight loss ( $F$

(3, 28) = 1.17,  $p > 0.05$ ). Furthermore, there were no significant effects of drug treatment on recovery of bladder control  $F(3, 28) = 0.43$ ,  $p > 0.05$ ; data not shown). Mortality was also unaffected, with only one death recorded in the vehicle group, and one in the 0.32  $\mu\text{mol}$  group. Only one case of spastic hypertonia was observed (0.01  $\mu\text{mol}$  group) for the entire experiment. Finally, although I observed a dose-dependent increase in autophagia, this was not a statistically significant effect ( $\chi^2 = 7.38$ ,  $p > 0.05$ ; data not shown).



*Figure 16: Effects of GR89696 on weight change across recovery.* This graph depicts the daily % weight loss across the 21-day recovery period, calculated by subtracting starting weight from daily weight. GR89696 did not affect weight change, or other signs of general health, following SCI. Results shown as Mean  $\pm$  S.E.M.

#### *Effects of GR89696 on tissue sparing at the site of injury*

Since GR89696 administration had a significant impact on locomotor recovery, I wanted to see whether the effects were due to decreased tissue sparing at the injury site. GR89696 produced similar behavioral outcomes at all doses tested, therefore only the

lowest (0.01  $\mu\text{mol}$ ) and the highest (0.32  $\mu\text{mol}$ ) dose groups were further assessed using histology. Spinal tissue was subdivided into rostral (1800 – 600  $\mu\text{m}$ ), center (0  $\mu\text{m}$ ), and caudal (-1800 – -2400  $\mu\text{m}$ ) segments. Four measures were analyzed: residual white matter, residual gray matter, tissue damage, and relative lesion (damage + missing tissue). There were no effects of drug dose on any of the histological measures at the center of the lesion (Figure 17). Similarly, there were no significant effects rostral to the lesion, although a main effect of drug dose on residual gray matter approached significance ( $F(2, 17) = 2.99, p = 0.077$ ). Caudally, however, I found a significant main effect of dose on residual gray matter ( $F(2, 15) = 5.74, p < 0.05$ ). Post-hoc analyses revealed that subjects in the 0.01  $\mu\text{mol}$  group had significantly less spared gray matter than subjects treated with vehicle. A main effect of drug dose also approached, but did not reach, significance for the measure of tissue damage caudal to the injury site ( $F(2, 15) = 2.98, p = 0.08$ ). There were no significant effects on residual white matter at this level of the spinal cord.

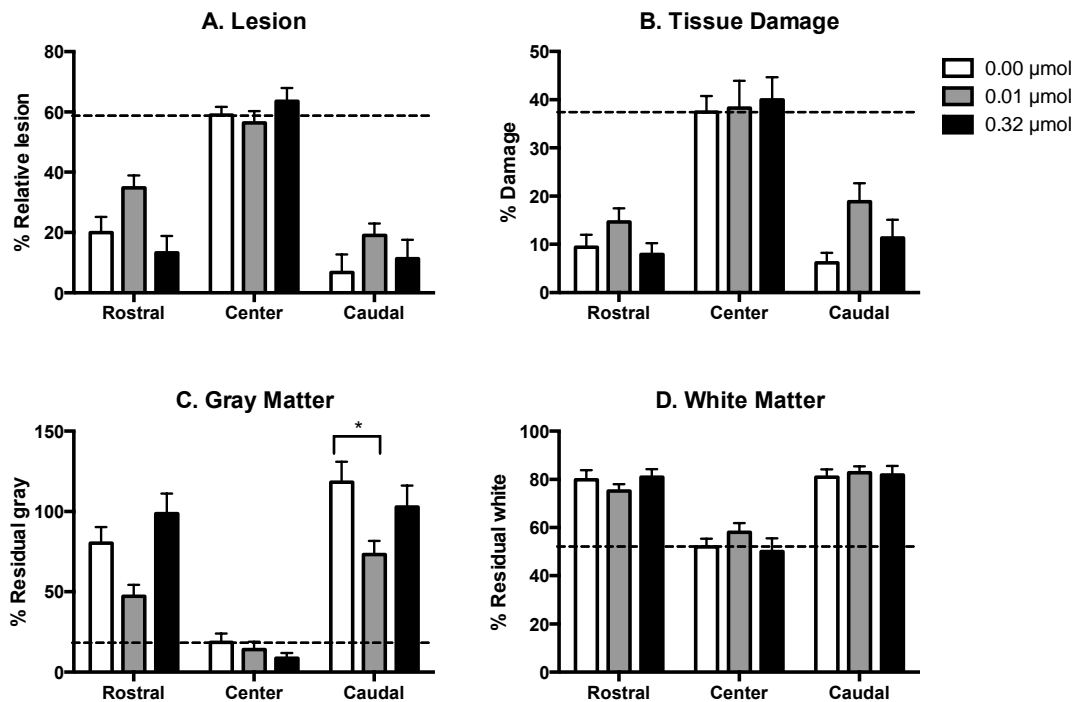


Figure 17: Effects of GR89696 on tissue sparing at the site of injury. At the end of the experiment, subjects were perfused and tissue was collected for histological analyses. The dose-dependent effects of GR89696 on lesion size (relative lesion), damage, residual gray matter and residual white matter are depicted. A single 0.01  $\mu\text{mol}$  dose of GR89696, administered in the acute phase of SCI, significantly decreased the amount of spared gray matter caudal to the injury center in comparison with vehicle-treated controls. Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests.

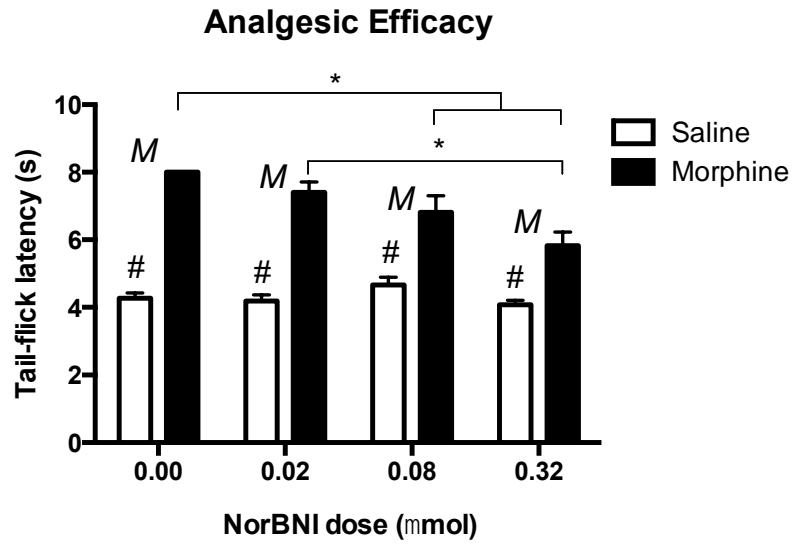
## Exp. 2B Necessity of KOR activation

In this experiment I used the highly selective KOR antagonist nor-Binaltorphimine (norBNI) to assess the necessity of KOR activation to the morphine-induced attenuation of function after SCI. To test whether antagonism of KORs blocks the adverse effects of morphine, subjects were pretreated with intrathecal norBNI (0, 0.02, 0.08, or 0.32  $\mu\text{mol}$  dissolved in distilled water). Two hours later, half of the

subjects in each dose group were treated with 90 µg of morphine and the remaining subjects were treated with vehicle (0.09% saline). The 2 hr waiting period between norBNI and morphine administration was chosen to account for the slow-onset of norBNI antagonistic action at the KOR, which reaches a plateau at 2 hr *in vivo* (Endoh et al., 1991). This 4 X 2 experimental design (8 groups) used a total of 100 rats (n=12 for doses 0.02, 0.08, 0.32 µmol norBNI; n=14 for dose 0 µmol norBNI).

#### *Effects of norBNI on the analgesic efficacy of morphine*

Sensory function was evaluated using the tail-flick test to establish baseline thermal reactivity thresholds. Prior to treatment, tail-flick scores ( $\pm$  S.E.M.) on day 1 ranged from  $3.79 \pm 0.21$  s to  $4.31 \pm 0.20$  s. Statistical analyses showed that there were no significant differences between the groups at this time point ( $F(7, 91) = 1.04$ ,  $p > 0.05$ ; data not shown). A two-factor ANOVA on post-treatment scores, however, revealed significant main effects of morphine ( $F(1, 91) = 193.97$ ,  $p < 0.05$ ) and norBNI treatment ( $F(3, 91) = 5.52$ ,  $p < 0.05$ ), and a significant interaction ( $F(3, 91) = 6.57$ ,  $p < 0.05$ ) on tail-flick latency. As shown in Figure 18, all groups that received morphine had increased tail-flick latencies relative to controls. NorBNI, however, decreased the analgesic effects of morphine, with a significant linear reduction in efficacy across increasing doses ( $F(1, 46) = 21.03$ ,  $p < 0.05$ ). Whereas rats that received morphine alone displayed maximal latencies (the test was automatically terminated at 8 seconds to prevent tissue injury), those pretreated with the highest dose of norBNI (0.32 µmol) had a mean tail-flick latency of  $5.83 \pm 0.40$  seconds.



*Figure 18: Effects of norBNI on the analgesic efficacy of morphine.* The analgesic efficacy of morphine challenged with norBNI pre-treatment is depicted for the test of thermal reactivity. All groups that received morphine had significantly increased tail-flick latencies relative to vehicle-treated controls. However, morphine's analgesic effects decreased with increasing doses of norBNI. Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests; #  $p < 0.05$  for planned comparisons; *M* = significant main effect of morphine.

#### *Effects of norBNI on locomotor recovery*

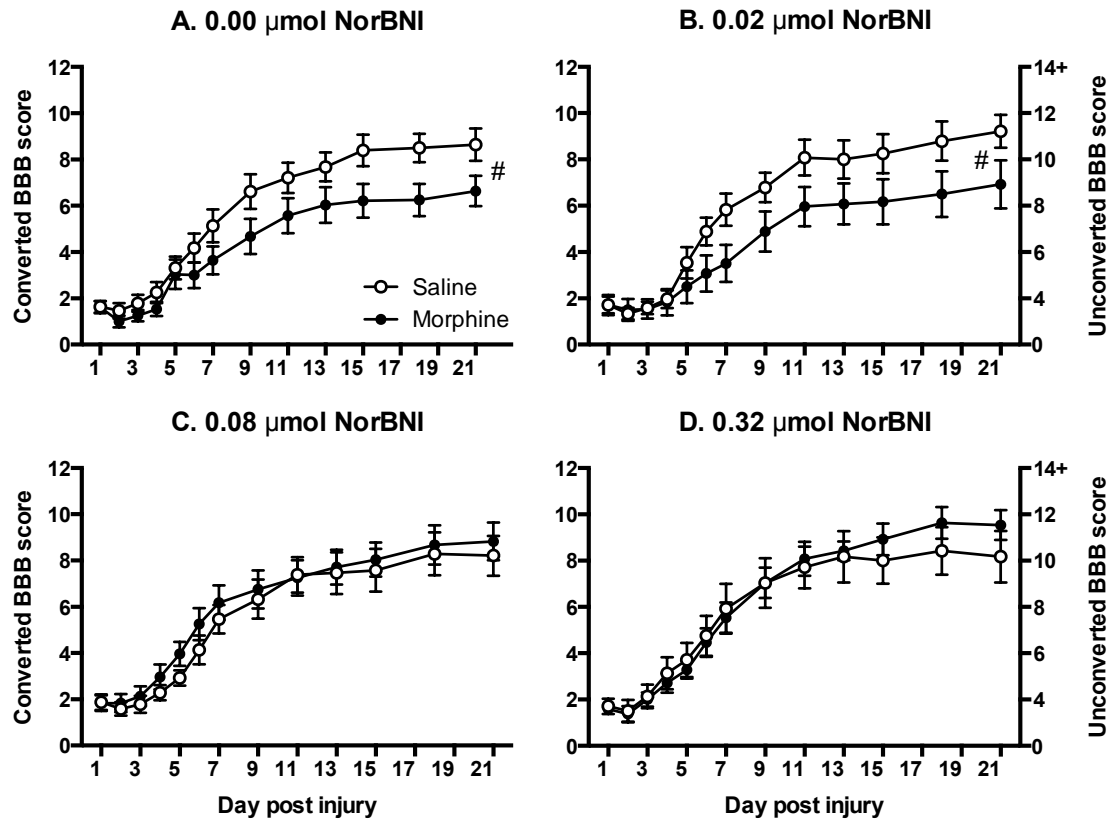
In addition to the effects on analgesia, I wanted to assess whether KOR activation was necessary to induce the morphine-induced attenuation of locomotor recovery (Hook et al., 2007; Hook et al., 2009; Hook et al., 2011; Woller et al., 2014; Woller et al., 2012). To address this, norBNI was administered as an adjuvant to morphine treatment 24 hours following SCI, and locomotor recovery was monitored for a 21-day period. Locomotor scores collected before treatment on day 1 did not differ significantly across groups ( $F(7, 92) = 0.01, p > 0.05$ ). Mean converted BBB scores ( $\pm$  S.E.M.) on day 1 ranged from  $1.61 \pm 0.26$  to  $1.88 \pm 0.33$ . A mixed-design ANCOVA



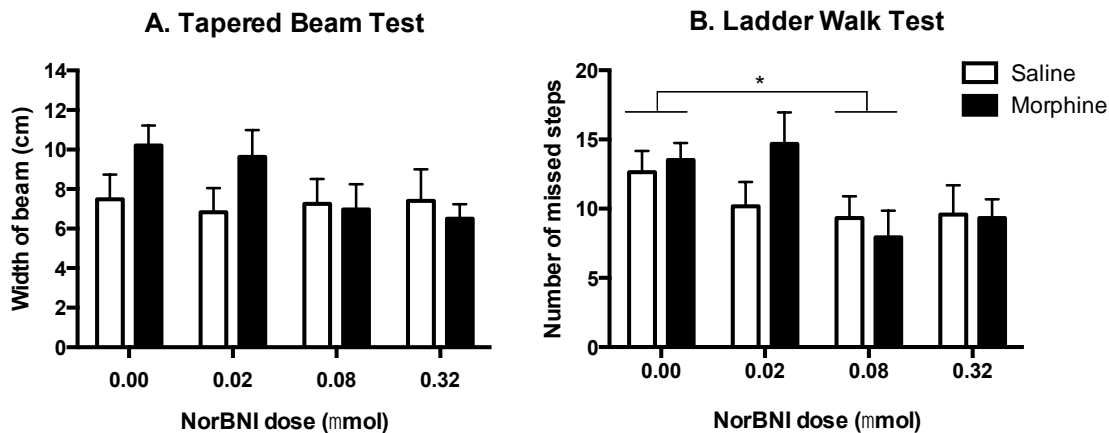
using day 1 scores as a covariate did not find significant main effects of morphine ( $F(1, 91) = 1.85, p < 0.05$ ) or norBNI treatment ( $F(3, 91) = 1.83, p > 0.05$ ). An interaction approached, but did not reach, significance ( $F(3, 91) = 2.30, p = 0.083$ ). However, as shown in Figure 19, planned comparisons revealed that control rats (0  $\mu\text{mol}$  of norBNI) treated with morphine showed significantly reduced locomotor recovery when compared to their vehicle-treated counterparts ( $F(1, 25) = 4.96, p < 0.05$ ), replicating our previous studies (Hook et al., 2009; Hook et al., 2011). Morphine and vehicle groups also differed significantly at the lowest dose (0.02  $\mu\text{mol}$ ) of norBNI ( $F(1, 21) = 5.78, p < 0.05$ ). In contrast, there were no differences between morphine- and vehicle-treated rats at the 0.08  $\mu\text{mol}$  ( $F(1, 21) = 0.46, p > 0.05$ ) or 0.32  $\mu\text{mol}$  ( $F(1, 21) = 0.17, p > 0.05$ ) doses of norBNI. This suggests that- although morphine and vehicle groups recovered equally when higher doses (0.08 and 0.32  $\mu\text{mol}$ ) of norBNI were used- lower doses of norBNI were not able to block the morphine-induced attenuation of locomotor recovery.

Motor recovery was further evaluated at the end of the 21-day recovery period using the tapered beam and ladder walk tests. Although we observed that morphine-treated rats at the higher doses of norBNI showed better performance on the tapered beam test than those receiving the lower doses (Figure 20), statistical analysis did not confirm significant main effects of norBNI ( $F(3, 92) = 1.17, p > 0.05$ ) or morphine ( $F(1, 92) = 1.62, p > 0.05$ ) treatment, and no interaction ( $F(3, 92) = 1.31, p > 0.05$ ). In the ladder walk test, I found a significant main effect of norBNI ( $F(3, 92) = 3.39, p < 0.05$ ). Post-hoc analysis showed that rats in the 0.08  $\mu\text{mol}$  dose performed better than rats not

treated with norBNI. However, I did not find an effect of morphine treatment ( $F(1, 92) = 0.60, p > 0.05$ ) or a significant interaction ( $F(3, 92) = 1.11, p > 0.05$ ).



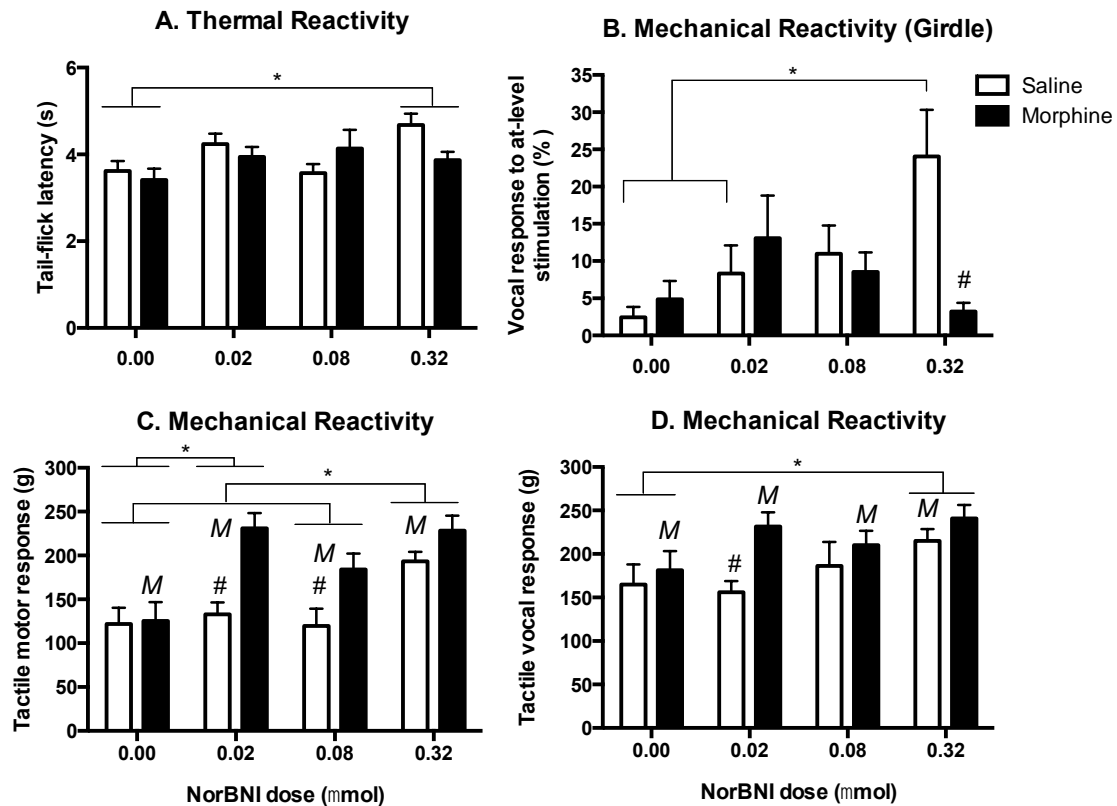
*Figure 19: Effects of norBNI on locomotor recovery.* NorBNI pretreatment blocked the adverse effects of morphine on long-term recovery of locomotor function. Morphine undermined recovery of locomotor function in the absence of norBNI (A) and at a dose of 0.02  $\mu\text{mol}$  norBNI (B). Pretreatment with 0.08 (C) or 0.32 (D)  $\mu\text{mol}$  norBNI, however, prevented the morphine-induced attenuation of locomotor recovery. Results shown as Mean  $\pm$  S.E.M. #  $p < 0.05$  for planned comparisons.



*Figure 20: Effects of norBNI on beam and ladder walk performance.* NorBNI improved motor performance on the ladder walk test conducted 21-days after injury. While there were no significant effects of morphine or norBNI on the tapered beam test (A), norBNI significantly improved performance on the ladder walk test (B). Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests.

#### *Effects of norBNI on sensory reactivity*

Long-term effects on sensory function were assessed at the end of the 21-day recovery period using the tail-flick, von Frey, and girdle tests. In the tail-flick test, mean latencies ( $\pm$  S.E.M.) ranged from  $3.41 \pm 0.26$  to  $4.68 \pm 0.26$  seconds. Although I did not find an effect of morphine ( $F(1, 92) = 0.95$ ,  $p > 0.05$ ) or an interaction ( $F(3, 92) = 2.05$ ,  $p > 0.05$ ), statistical analyses revealed a significant main effect of norBNI treatment ( $F(3, 91) = 2.95$ ,  $p < 0.05$ ) on thermal reactivity after day 21. As shown in Figure 21, norBNI improved tail-flick latency. In effect, post-hoc tests showed that rats treated with the highest dose of norBNI ( $0.32 \mu\text{mol}$ ) had significantly higher reactivity thresholds than those that did not receive norBNI treatment ( $0.00 \mu\text{mol}$ ).



**Figure 21: Effects of norBNI on sensory reactivity.** There were significant effects of norBNI pre-treatment on the long-term recovery of sensory function. At the end of the 21-day recovery period, there was a significant main effect of norBNI on thermal reactivity (A). NorBNI decreased thermal reactivity relative to subjects that were not given norBNI. By contrast, on the girdle test of at-level allodynia, there was a significant interaction between norBNI and morphine treatment (B); norBNI appeared to increase reactivity in subjects that were not treated with morphine. As found on the test of thermal reactivity, both morphine and norBNI decreased motor (C) and vocal responses (D) responses to tactile stimulation at day 21 post-injury. Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests; #  $p < 0.05$  for planned comparisons; *M* = significant main effect of morphine.

Mechanical reactivity of the hindpaws tested at the end of the recovery period appeared to decrease with higher doses of norBNI. A two-factor ANOVA on motor thresholds uncovered significant main effects of morphine ( $F(1, 91) = 14.82, p < 0.05$ )

and norBNI ( $F(3, 91) = 8.58, p < 0.05$ ), but an interaction only approached significance ( $F(3, 91) = 2.47, p = 0.067$ ). Across norBNI groups, rats treated with higher doses of norBNI showed increased mechanical thresholds. Rats that did not receive norBNI showed the most reactivity to tactile stimulation, and significantly differed from those in the 0.02  $\mu\text{mol}$  and 0.32  $\mu\text{mol}$  groups. On the other hand, rats treated with the highest dose of norBNI (0.32  $\mu\text{mol}$ ) showed the least reactivity in this test. Surprisingly, overall, vehicle-treated rats had significantly lower motor thresholds than morphine-treated animals ( $p < 0.05$ ). Similarly, a significant main effect of morphine treatment on vocal thresholds to von Frey stimulation ( $F(1, 91) = 6.10, p < 0.05$ ) supported the observation that vehicle-treated rats exhibited more symptoms of mechanical hyperalgesia in the long term. An effect of norBNI treatment approached but did not reach significance ( $F(3, 92) = 2.55, p = 0.06$ ), and no interaction was observed ( $F(3, 92) = 0.88, p > 0.05$ ). Together, the motor and vocal responses obtained in the von Frey test of mechanical reactivity suggest that morphine treatment in combination with norBNI, may reduce pain and prevent the development of allodynia following SCI.

Lastly, in the girdle test of at-level allodynia, I did not find main effects of norBNI ( $F(3, 92) = 2.48, p > 0.05$ ) or morphine ( $F(1, 92) = 2.15, p > 0.05$ ), but there was a significant interaction between the two treatments ( $F(3, 91) = 4.37, p < 0.05$ ). As depicted in Figure 21, vocal responses to at-level stimulation decreased with escalating norBNI doses for morphine-treated rats. On the other hand, higher doses of norBNI appeared to exacerbate allodynia in vehicle-treated controls, shown by a steady increase

in vocalization. At the highest dose tested (0.32  $\mu$ mol), vehicle-treated rats vocalized approximately 24% of the time, compared to 3% in morphine-treated rats.

#### *Effects of norBNI on general health*

As in the previous experiments, weight was monitored throughout recovery as an index of general health. Weight immediately before injury differed across groups ( $F(7, 90) = 2.96, p < 0.05$ ; data not shown). Starting weight ( $\pm$  S.E.M.) across dose groups were the following: for 0  $\mu$ mol norBNI, 358.14 $\pm$ 7.95 g for saline-treated subjects and 348.79  $\pm$ 5.99 g for those treated with morphine; for 0.02  $\mu$ mol norBNI, 348.25 $\pm$ 4.25 g for saline and 336.36 $\pm$ 3.88 g for morphine; for 0.08  $\mu$ mol norBNI, 329.58 $\pm$ 5.04 g for saline and 342.00 $\pm$ 4.74 g for morphine; and for 0.32  $\mu$ mol norBNI, 331.60 $\pm$ 3.93 g for saline and 341.58 $\pm$ 3.53 g for morphine. To assess changes in weight across recovery taking into account initial differences, the daily % weight loss was calculated by subtracting the starting weight (weight on the day of surgery) from daily weight. A repeated-measures ANOVA on daily % weight loss values revealed that weight across recovery was unaffected by morphine ( $F(1, 90) = 0.48, p > 0.05$ ) or norBNI administration ( $F(3, 90) = 1.57, p > 0.05$ ), with no significant interaction ( $F(3, 90) = 1.34, p > 0.05$ ). As shown in Figure 22, weight change across recovery was virtually the same for morphine- and saline-treated subjects at all doses of norBNI. Subjects not treated with norBNI, however, showed significantly attenuated weight gain after injury if treated with morphine ( $F(1, 26) = 5.55, p < 0.05$ ). This suggests that norBNI treatment, aside from blocking morphine-induced attenuation of locomotor recovery, also had protective effects on general health following SCI.

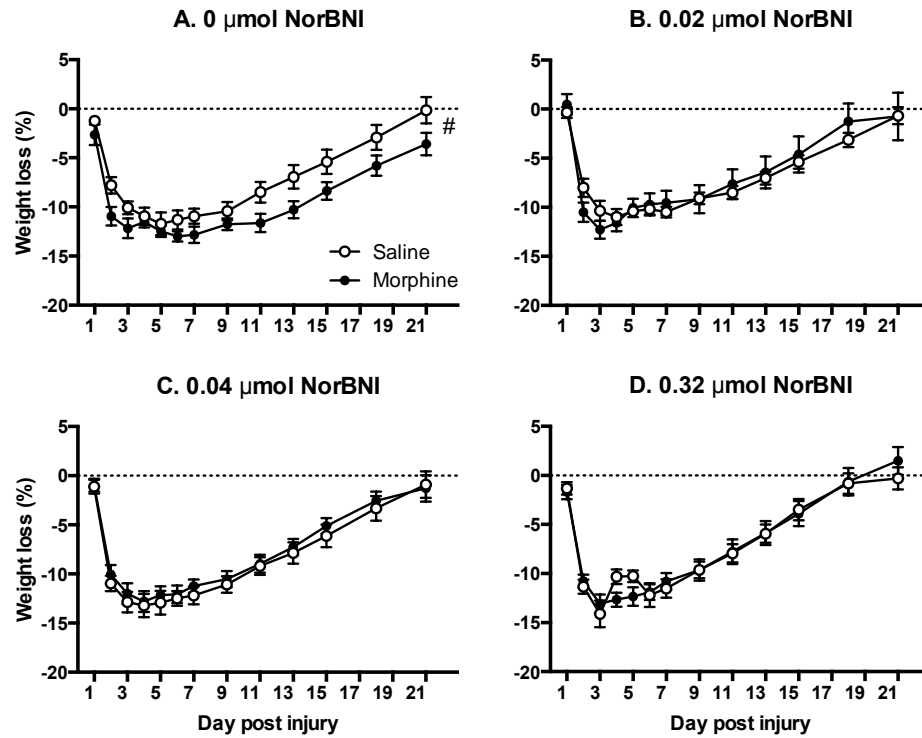


Figure 22: Effects of norBNI on weight change across recovery. Weight loss was unaffected by morphine or norBNI treatment. Results shown as Mean  $\pm$  S.E.M. #  $p < 0.05$  for planned comparisons.

There were very few instances of mortality, autophagia, and spasticity overall. In the entire experiment, only one rat had to be replaced due to mortality (treated with 0.02  $\mu$ mol norBNI and morphine). Three rats exhibited mild autophagia, which resolved rapidly with treatment and did not require removal from the study (all were in the 0.02  $\mu$ mol group; one treated with vehicle, and two with morphine). Lastly, four rats developed spastic hypertonia during the recovery period (two in the 0.08  $\mu$ mol group,

and one each in the 0 and 0.02  $\mu\text{mol}$  groups, and all received morphine). No other complications were observed.

#### *Effects of norBNI on tissue sparing at the site of injury*

To assess whether the effects of the high doses of norBNI on locomotor function were the result of increased neuroprotection, I assessed the amount of tissue sparing in the spinal cord at the end of the recovery period. Spinal tissue was subdivided into rostral (2400 – 600  $\mu\text{m}$ ), center (0  $\mu\text{m}$ ), and caudal (-600 – -2400  $\mu\text{m}$ ) segments. Four measures were analyzed: relative lesion size (damage + missing tissue), tissue damage, residual gray matter, and residual white matter.

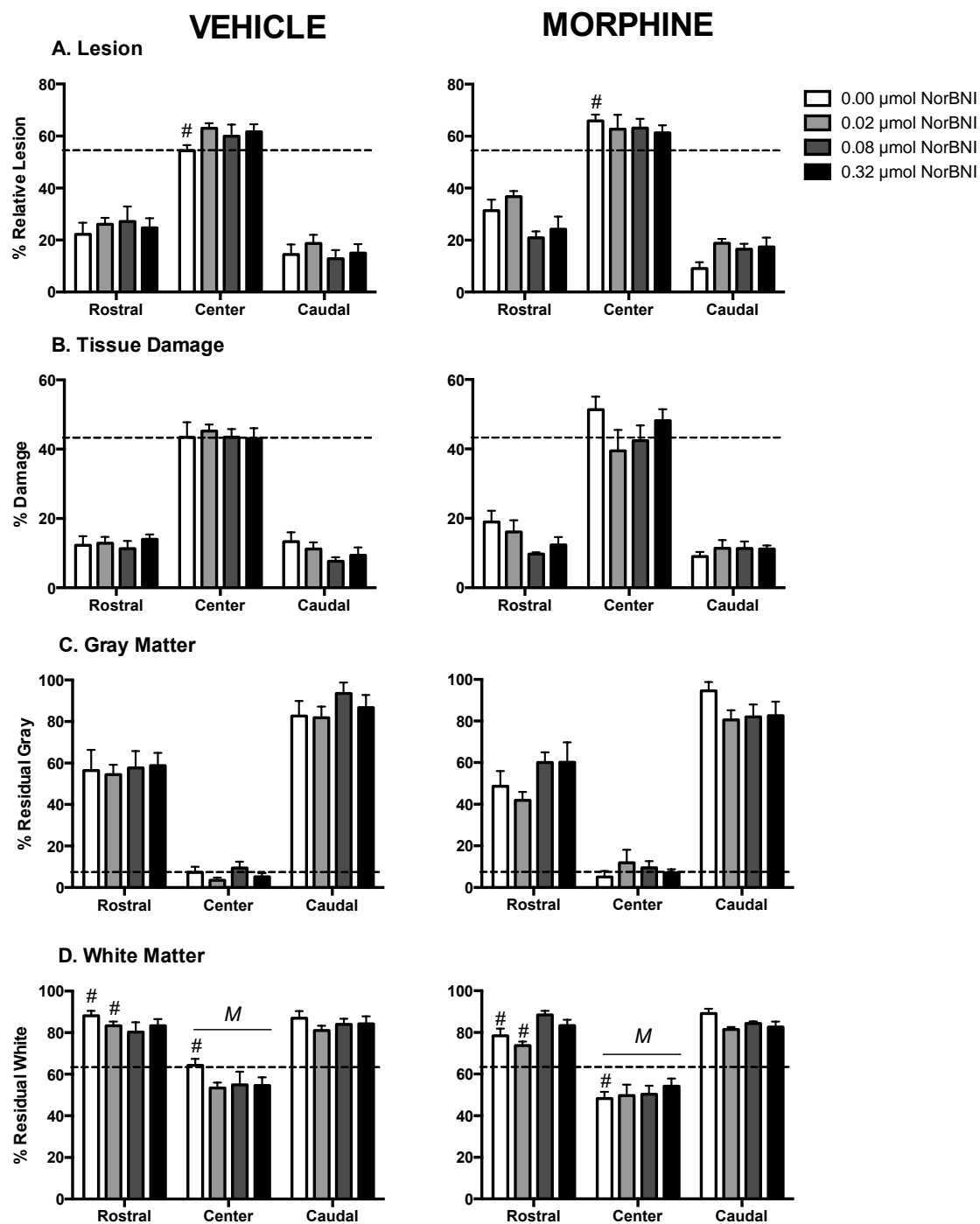
There were no significant main effects of norBNI or morphine treatment on relative lesion size across the extent of the spinal cord (Figure 23). Planned comparisons, however, revealed a significant difference between vehicle and morphine-treated rats without norBNI pre-treatment at the center of the lesion ( $p < 0.05$ ). Morphine-treated rats had increased damage at the injury center, with a mean lesion size ( $\pm$  S.E.M.) of  $3.12 \pm 0.26 \text{ mm}^2$  compared to  $2.39 \pm 0.23 \text{ mm}^2$  for vehicle controls. Similarly, no main effects on damage were observed, although rostral to the injury planned comparisons showed that a difference between vehicle and morphine rats at the 0  $\mu\text{mol}$  dose of norBNI approached significance ( $p = 0.066$ ).

None of the groups differed on the percentage of residual gray matter at any level of the spinal cord. However, for residual white matter, the treatment groups showed extensive differences in the center and rostral segments. I found a significant main effect of morphine treatment at the center of the lesion ( $F(1, 56) = 3.94$ ,  $p < 0.05$ ), with



morphine-treated rats showing less residual white matter than their vehicle-treated counterparts, irrespective of norBNI. Planned comparisons also uncovered a significant difference between vehicle and morphine controls (0  $\mu\text{mol}$  norBNI) at this level ( $2.48 \pm 0.12 \text{ mm}^2$  and  $1.86 \pm 0.12 \text{ mm}^2$  respectively). Rostral to the injury site, the effects on residual white matter appeared to be the result of a significant interaction between norBNI and morphine treatment ( $F(3, 56) = 3.75, p < 0.05$ ). At the lower doses of norBNI (0 and 0.02  $\mu\text{mol}$ ), morphine-treated rats lost more white matter than vehicle-treated rats, and these differences reached statistical significance ( $p < 0.05$ ). At the 0  $\mu\text{mol}$  dose, residual white matter was  $3.39 \pm 0.10 \text{ mm}^2$  for the vehicle group, and  $3.02 \pm 0.21 \text{ mm}^2$  for the morphine group. At 0.02  $\mu\text{mol}$ ,  $3.21 \pm 0.20 \text{ mm}^2$  for vehicle, and  $2.84 \pm 0.25 \text{ mm}^2$  for morphine. Lastly, at the 0.08  $\mu\text{mol}$  dose, a difference between morphine and vehicle groups approached, but did not reach significance ( $p = 0.073$ ), and no differences were found at the 0.32  $\mu\text{mol}$  dose ( $p = 0.98$ ).

*Figure 23: Effects of norBNI on tissue sparing at the site of injury.* As found in previous studies, morphine increased the percent relative lesion at the injury center (0  $\mu\text{m}$ ) relative to vehicle treated controls. This effect was not present in morphine-treated subjects that were pre-treated with norBNI. There were no main effects of morphine or norBNI on relative lesion size (A), tissue damage (B), or residual gray matter (C). However, residual white matter was significantly affected by morphine treatment at the center of the lesion, and there was a significant interaction between norBNI and morphine treatment rostral to the injury site (D). Morphine decreased the residual white matter at the center of the lesion, but in the rostral segment, norBNI pre-treatment at higher doses blocked the adverse effects of morphine on white matter sparing. Results shown as Mean  $\pm$  S.E.M. #  $p < 0.05$  for planned comparisons;  $M$  = significant main effect of morphine.



## Discussion

The experiments presented here evaluated the contributions of the MOR and KOR systems to the morphine-induced attenuation of function following SCI. First, I assessed the contribution of the MOR system using the selective agonist DAMGO. At doses commensurate with an effective dose of morphine, DAMGO produced robust analgesia in tests of thermal and mechanical reactivity, without producing any adverse effects on recovery (Experiment 1.A). To further explore the role of the MOR following SCI, I challenged morphine treatment with the highly-selective antagonist CTOP (Experiment 1.B). At 10 times the inhibitory constant, 10  $\mu\text{g}$  of CTOP reduced, but did not completely block, the analgesic effects of morphine. Similarly, CTOP did not effectively block the adverse effects of morphine. Instead of improving recovery for morphine-treated subjects, CTOP alone appeared to impair recovery of function.

Next, I used a similar approach to assess the contribution of the KOR system following SCI. Despite its analgesic efficacy, treatment with the selective KOR-agonist GR89696 significantly undermined recovery of locomotor function, increased mechanical reactivity, and decreased gray matter sparing after SCI (Experiment 2.A). In fact, the lowest dose of GR89696 replicated the negative side effects associated with morphine treatment in our previous studies (Hook et al., 2009; Hook et al., 2011)- even at a dose 32 times lower than the 0.32  $\mu\text{mol}$  (90  $\mu\text{g}$ ) dose of morphine. Further supporting a role of KOR activation in the morphine-induced attenuation of function, pretreatment with the KOR-antagonist norBNI significantly reduced the adverse effects of morphine treatment (Experiment 2.B). In the absence of norBNI, morphine-treated

rats showed decreased locomotor recovery relative to vehicle-treated controls, replicating our previous studies (Hook et al., 2009; Hook et al., 2011). However, moderate to high doses of norBNI (0.08 and 0.32  $\mu$ mol respectively), blocked the adverse effects of morphine on locomotor recovery, improved overall health, and increased white matter sparing at the lesion site, while maintaining moderate analgesia.

Based on the data presented here, I propose that the analgesic efficacy of intrathecal morphine in the SCI model is mediated by binding to MORs and KORs. While studies on opioid receptor distribution in the spinal cord support the significant contribution of the MOR to the antinociceptive effects of morphine (Arvidsson et al., 1995; Mansour et al., 1995b), the results of selective activation of the KOR were unexpected. Studies have long disputed the role of the KOR in spinal analgesia. For example, in a study by Leighton and colleagues (1988), intrathecal administration of three different KOR agonists (PD1 17302, U50488, U69593) failed to increase antinociception to noxious mechanical and thermal stimuli, even at doses up to 100  $\mu$ g. Others have suggested that KOR-mediated analgesia is intensity- and stimulus-dependent, with intrathecally applied KOR ligands showing potency on tests of chemical-visceral pain but not cutaneous-thermal or electrical sensory input (Millan, 1989; Schmauss, 1987; Schmauss & Yaksh, 1984). Furthermore, intrathecal administration of exogenous and endogenous KOR ligands has also been associated with paralysis and flaccidity of the tail and hind limbs, effects which complicate the interpretation of analgesic tests requiring a motor response (Herman & Goldstein, 1985; Leighton et al., 1988).

In contrast to these studies, however, others have shown that KOR agonists can produce significant spinal analgesia (Lahti et al., 1982; Piercey et al., 1982). Indeed, using knockout mice, Yamada et al. (2006) found that intrathecal administration of morphine produced analgesia in the absence of MORs by acting through spinal KORs. In our studies, this could explain why morphine- the prototypical MOR agonist- produced moderate analgesia following treatment with CTOP, a potent and selective MOR antagonist. Further, Yamada's findings could also explain why the selective KOR agonist, GR89696, exhibited an analgesic profile comparable to morphine and DAMGO. Acute intrathecal administration of GR89696 increased subjects' thermal and mechanical thresholds. Notably, I verified that the decreased motor responses in these tests were not simply due to paralysis by recording vocal responses to stimulation. Subjects treated with GR89696 displayed increased vocal response thresholds with mechanical stimulation, indicative of analgesia. Overall, these findings point to an important role for the spinal KOR system in intrinsic pain modulation after SCI.

Regarding the effects on recovery of function, as discussed in the introduction to this chapter, the literature strongly implicates the KOR system in the pathophysiology of SCI. There is controversy, however, regarding the opioid-receptor mediated (classic) and non-opioid (non-classic) actions of KOR ligands. Supporting a KOR-mediated mechanism, previous studies have shown that administration of norBNI limits the paralytic effects of dynorphin (Faden, 1990), and has been shown to improve functional outcomes in rodent models of spinal (Faden, 1990; Faden et al., 1987) and brain (Vink et al., 1991) injury. Conversely, although showing decreased potency, administration of

Dyn A-(2-17), a prodynorphin product inactive at opioid receptors, replicates dynorphin's adverse effects on locomotor function (Faden, 1990). It has been posited that these pathophysiological effects may result from NMDA-mediated excitotoxicity. NMDA antagonists limit dynorphin-induced behavioral effects (Bakshi & Faden, 1990a, 1990b; Caudle & Isaac, 1988). Moreover, dynorphin administration increases extracellular levels of excitatory amino acids, including glutamate and aspartate, through a non-classic mechanism (Faden, 1992). These studies suggest that while KOR activation may contribute to decreased locomotor function after SCI, endogenous and exogenous opioids may also engage alternative mechanisms that compromise recovery. Although our findings strongly support a KOR-mediated mechanism of action, the non-classic effects of morphine and other opioids should be further evaluated.

In conclusion, I have shown that KOR activation with GR89696 is sufficient to undermine locomotor recovery following SCI. I have also shown that blocking this opioid receptor system using norBNI prevents morphine's adverse effects. In contrast, DAMGO did not affect recovery, and CTOP did not effectively block the negative side-effects of morphine. Based on these data, I hypothesize that the adverse effects of morphine on recovery of locomotor function are mediated by the KOR system. Importantly, given that frequently prescribed analgesics like oxycodone act through the KOR receptor system, it is paramount that I identify the mechanisms mediating the adverse effects of KOR activation after SCI. Indeed, our data demonstrate that even very low doses of KOR ligands may undermine recovery, as shown by the attenuation of function observed at a dose 32-fold lower than an effective dose of morphine. Lastly, the

role of the MOR should not be discounted. Although I did not observe any effects of DAMGO after a single intrathecal injection, it is possible that detrimental effects may emerge with prolonged administration (Mao et al., 1995). Alternative mechanisms, including non-classic opioid receptor signaling, also need to be further explored. Ultimately, elucidating the molecular mechanisms underlying the effects of morphine, and other opioids, is imperative in order to develop pharmacological interventions that are both safe and efficacious in a clinical setting.

## CHAPTER IV

### EVALUATION OF THE NON-CLASSIC OPIOID RECEPTORS\*

#### Introduction

In the previous chapter, I evaluated the contribution of the classic opioid receptors to the morphine-induced attenuation of function following SCI. Using selective agonists and antagonists, I assessed the differential effects of the MOR and KOR spinal systems. The results of these experiments support a role for the KOR, but not the MOR, in the adverse effects of morphine administration following SCI. However, as already discussed in Chapter 1, although morphine binds to the classic opioid receptors with high affinity, it may also bind to other receptors in non-classic fashion (Kristensen et al., 1994; Wang et al., 2012). In this chapter, I will assess the role of non-classic opioid receptor activation in the attenuation of recovery observed when morphine is administered following trauma.

Non-classic opioid receptors are sites that can bind opioids in a non-stereoselective fashion (Goldstein et al., 1971). As opposed to the classic opioid receptors, these sites accept natural and unnatural opioids (opioid enantiomers) and do not result in analgesia. The extant literature suggests that the toll-like receptors (TLRs) are the primary sites of non-classic opioid binding (Hutchinson et al., 2010a; Hutchinson

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\* Part of the data reported in this chapter is reprinted from Aceves, M., Mathai, B.B., & Hook, M.A. (2016). Evaluation of the effects of specific opioid receptor agonists in a rodent model of spinal cord injury. *Spinal Cord*, 54(10), 767-777 (Copyright 2016 by the authors).



et al., 2008b; Hutchinson et al., 2010b; Kigerl et al., 2007; Lewis et al., 2010). The toll-like receptors (TLRs) are important components of the innate immune system (Kawasaki & Kawai, 2014). These receptors form part of a wide class of pattern recognition receptors (PRRs) that detect danger signals in the cellular environment, including pathogen-associated microbial patterns (PAMPs) and danger-associated molecular patterns (DAMPs) released from dying cells. Ultimately, TLR binding triggers signaling cascades that activate kinases and transcription factors, and eventually result in the production and release of pro-inflammatory cytokines, chemokines, and other molecules.

I hypothesize that morphine may exert its negative effects through activation of these non-classic opioid receptors found on immune-competent cells (Hutchinson et al., 2007; 2008b; Tawfik et al., 2005; Watkins et al., 2009). Indeed, research suggests that opioid ligands can bind to TLR4 (the receptor that recognizes lipopolysaccharide) and its accessory molecules in a non-stereoselective manner, leading to glial activation and initiating the inflammatory response (Hutchinson et al., 2010a; Hutchinson et al., 2010b; Lewis et al., 2010; Wang et al., 2012). The subsequent release of pro-inflammatory cytokines in the spinal cord has been shown to oppose opioid analgesia and facilitate pain (Hutchinson et al., 2008a; Johnston et al., 2004). Importantly, in our SCI model, we have shown that morphine administration results in increased pro-inflammatory cytokine expression at the injury site, and that blocking the interleukin-1 (IL-1) receptor during treatment with this analgesic prevents the morphine-induced attenuation of locomotor recovery (Hook et al., 2011). These data suggest that by binding to non-classic opioid

receptors, morphine may alter the normal immune response following SCI, resulting in the adverse long-term consequences typically observed in this model.

To address this, the following experiments evaluated the contribution of non-classic opioid receptor activation to the morphine-induced attenuation of function following SCI. Since the extant literature strongly supports a role of TLR4 in the non-classic effects of morphine administration, I first assessed the role of this receptor using the selective agonist LPS and selective antagonist LPS-RS. Next, for a more thorough investigation of non-classic opioid receptor binding, I used the unnatural enantiomer of morphine ([+]- morphine). This compound cannot bind to the stereoselective classic opioid receptors, but may bind to other non-classic receptor sites (including the TLRs). Finally, since I hypothesized that binding to non-classic receptors ultimately leads to glial activation, I challenged morphine treatment with minocycline, a glial inhibitor. Our results showed that neither administration of LPS or [+]- morphine replicated the adverse effects of morphine. However, inhibiting the innate immune response with LPS-RS or minocycline blocked the adverse effects of morphine administration, without disrupting the analgesic efficacy of the drug. This suggests that following injury, opioid-immune interactions mediate the analgesic and pathological responses to morphine.

## **Methods and results**

### *The role of the toll-like receptor 4*

#### **Exp. 3A Sufficiency of TLR4 activation**

In this experiment I used the natural TLR4 agonist LPS (Lipopolysaccharide from *Escherichia coli*; Sigma-Aldrich, St. Louis, MO, USA) to assess the contribution of

this non-classic opioid receptor to the morphine-induced attenuation of function after SCI. To test whether increased activation of TLR4 following SCI is sufficient to reproduce the adverse effects of morphine, LPS was dissolved in endotoxin-free water. The solution was then administered via an intrathecal catheter, as described in Chapter II General Methodology. Based on previous literature, a high dose of LPS was tested (100  $\mu$ g) along with a vehicle control (0  $\mu$ g). With an  $n=9$  per group, this experimental design used a total of 18 rats.

#### *Analgesic efficacy of LPS*

Sensory function was evaluated using the tail-flick test to establish baseline thermal reactivity thresholds. Prior to treatment, tail-flick scores ( $\pm$  S.E.M.) were  $4.00 \pm 0.17$  s and  $3.75 \pm 0.34$  s for the vehicle and LPS groups, respectively. Statistical analyses showed that there were no significant differences between the groups at this time point ( $F(1, 16) = 0.40$ ,  $p > 0.05$ ; data not shown). As expected, selective activation of TLR4 using LPS did not result in analgesia ( $F(1, 16) = 2.36$ ,  $p > 0.05$ ; Figure 24).

Although analgesia was not expected following LPS administration, hyperalgesia has been reported in the literature (Reeve et al., 2000). Hyperalgesia was not observed in the injured subjects receiving 100  $\mu$ g LPS. This could have been due to a masking effect by the injury itself, or could indicate that our LPS batch was defective. To ascertain that the LPS was working properly, I ran an acute analgesia study using sham subjects. Sham subjects were administered 100  $\mu$ g LPS or vehicle. An hour later, all subjects were

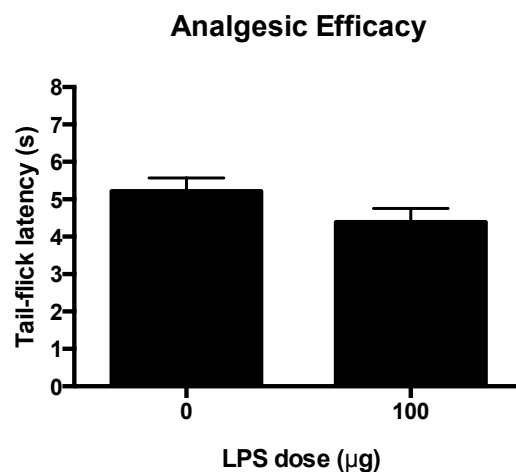


Figure 24: Analgesic efficacy of LPS. The results of the tail-flick test of thermal reactivity are depicted. Administration of LPS to the spinal cord did not produce analgesia. Results shown as Mean  $\pm$  S.E.M.

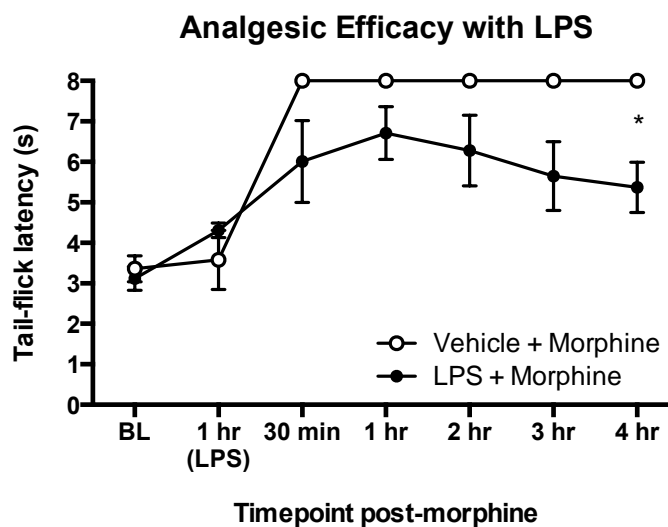


Figure 25: Analgesic efficacy of morphine with LPS. Sham subjects were treated with vehicle or LPS, followed by morphine treatment. Vehicle controls showed robust analgesia at all testing points following morphine administration. LPS-treated subjects did not reach full analgesia, and efficacy began to decrease at 2 hr post-morphine. Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$

treated with 90  $\mu$ g morphine, and analgesic efficacy was measured for the next 4 hours using the tail-flick test. As shown in Figure 25, both groups showed increased tail-flick latency following morphine administration. However, as opposed to the vehicle-treated subjects, subjects treated with LPS and morphine did not display signs of robust analgesia (did not reach an 8 s tail-flick latency). Moreover, although I did not observe decreases in analgesic efficacy at 4 hrs in the vehicle controls, LPS-treated subjects also showed decreases in analgesia starting at 2 hrs, and significant behavioral differences by 4 hrs ( $p < 0.05$ ). These results indicate that the LPS was working properly.

#### *Effects of LPS on locomotor recovery*

Surprisingly, LPS did not have a significant effect on recovery of locomotor function (Figure 26). Mean converted BBB scores ( $\pm$  S.E.M.) collected before treatment on day 1 were  $2.39 \pm 0.46$  for vehicle-treated controls and  $2.22 \pm 0.44$  for LPS-treated subjects, and did not differ statistically ( $F(1, 16) = 0.06$ ,  $p > 0.05$ ). Using day 1 scores as a covariate, a mixed-design ANOVA revealed no group differences in locomotor function across the recovery period ( $F(1, 15) = 0.77$ ,  $p > 0.05$ ). Furthermore, as shown in Figure 27, there were no significant differences between the groups after day 21 on either the beam ( $F(1, 16) = 1.01$ ,  $p > 0.05$ ) or ladder ( $F(1, 16) = 0.08$ ,  $p > 0.05$ ) tests.

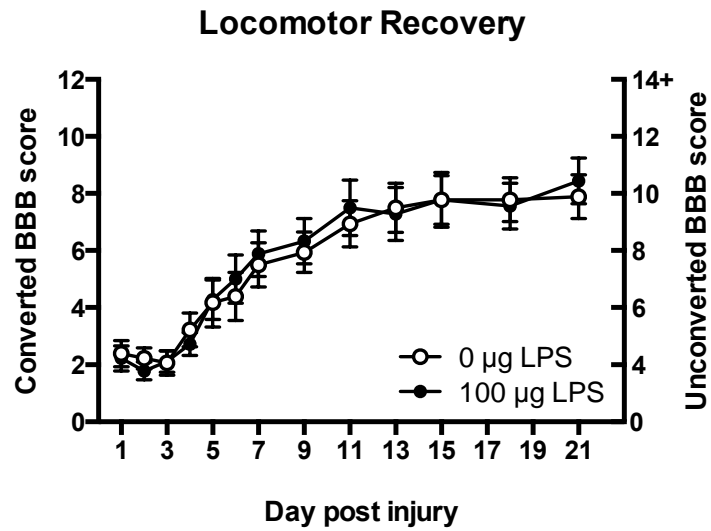


Figure 26: *Effect of LPS on locomotor recovery.* Subjects were monitored for 21 days following a moderate contusion SCI. Intrathecal administration of LPS, on day 1 post-injury, did not affect locomotor recovery assessed with the BBB scale. Results shown as Mean  $\pm$  S.E.M.

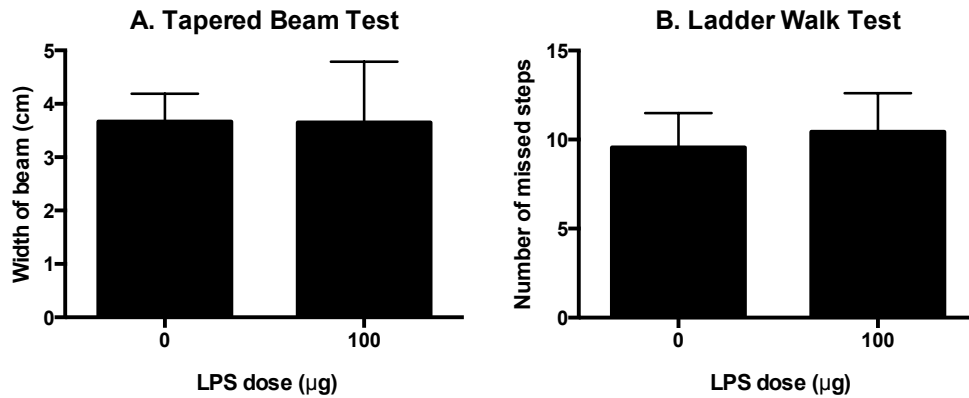
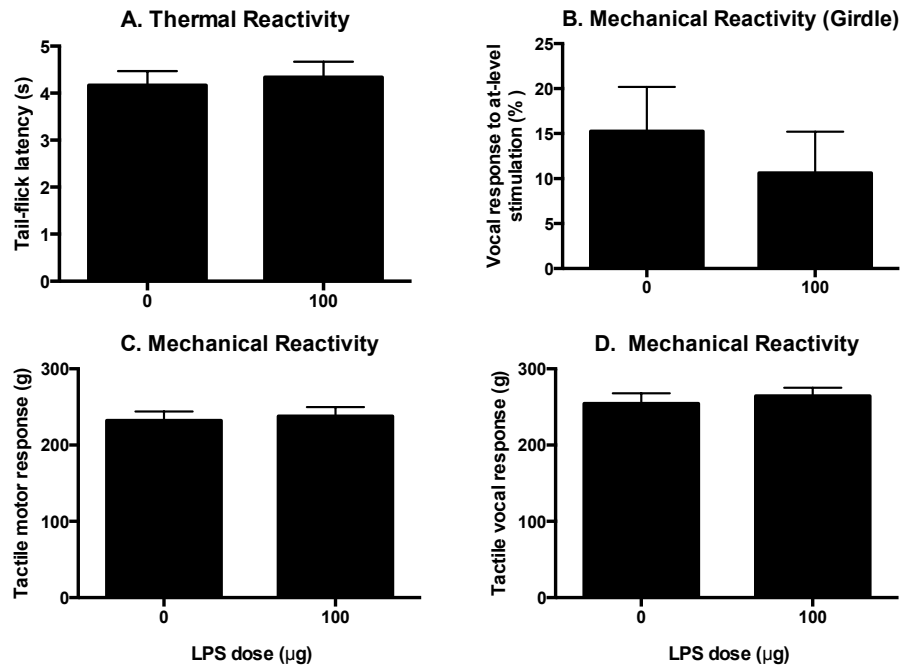


Figure 27: *Effects of LPS on beam and ladder walk performance.* At the end of the 21-day recovery period, locomotor recovery was further assessed using the tapered beam (A) and ladder walk (B) tests. Administration of LPS following SCI did not affect performance on either task. Results shown as Mean  $\pm$  S.E.M.

### *Effects of LPS on sensory reactivity*

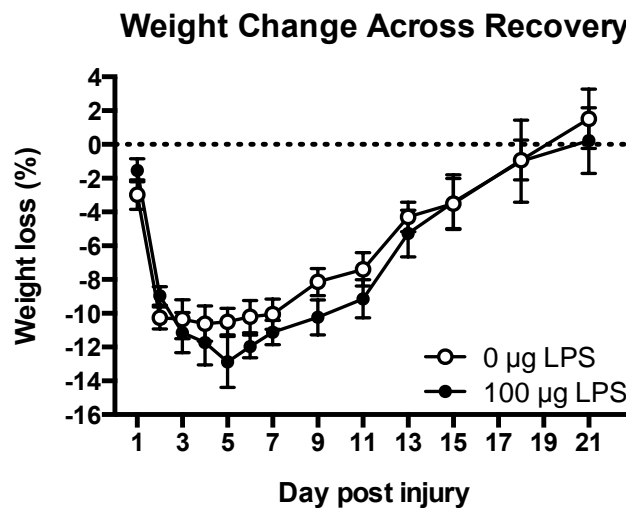
Sensory function was re-assessed at the end of the 21-day recovery period using the tail-flick, von Frey, and girdle tests (Figure 28). As observed with locomotor recovery, treatment with LPS did not affect sensory reactivity after day 21 post-injury. The groups did not show any differences in thermal reactivity assessed with the tail-flick test ( $F(1, 16) = 0.13, p > 0.05$ ). Furthermore, no differences were observed on motor ( $F(1, 16) = 0.10, p > 0.05$ ) or vocal reactivity ( $F(1, 16) = 0.30, p > 0.05$ ) to a mechanical stimulus applied to the hindpaws. Finally, administration of LPS did not affect at-level allodynia assessed with the girdle test ( $F(1, 14) = 0.23, p > 0.05$ ).



*Figure 28: Effects of LPS on sensory reactivity.* At the end of the 21-day recovery period, sensory reactivity was assessed using the tail-flick (A), girdle (B), and von Frey (C, D) tests. LPS administration did not result in the development of paradoxical pain. Results shown as Mean  $\pm$  S.E.M.

### *Effects of LPS on general health*

Weight was monitored as an index of general health. Weight immediately before injury did not differ across groups ( $F(1, 16) = 0.002, p < 0.05$ ; data not shown). To assess changes in weight across recovery, the daily % weight loss was calculated by subtracting the starting weight (weight at the day of surgery) from daily weight. A repeated-measures ANOVA on daily % weight loss values showed that there were no significant differences between the groups ( $F(1, 16) = 0.32, p > 0.05$ ; Figure 29).



*Figure 29: Effect of LPS on weight change across recovery.* This graph depicts the daily % weight loss across the 21-day recovery period, calculated by subtracting starting weight from daily weight. LPS did not affect weight change, or other signs of general health, following SCI. Results shown as Mean  $\pm$  S.E.M.

In addition to weight, mortalities, autophagia, spastic hypertonia, and recovery of bladder control were also recorded to assess general health. There were no mortalities and none of the subjects showed signs of spastic hypertonia in this experiment. Further, I



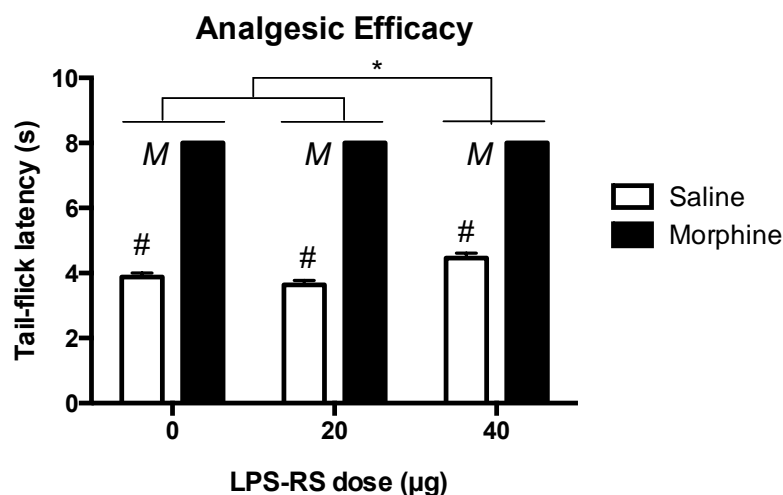
did not observe any significant effects of drug treatment on bladder control ( $F(1, 16) = 0.82$ ,  $p < 0.05$ ; data not shown). Lastly, treatment did not exacerbate autophagia. Only two subjects showed signs of self-harm, one in each of the groups.

### **Exp. 3B Necessity of TLR4 activation**

While LPS administration did not replicate the morphine effect observed in our previous studies, I hypothesized that the downstream effects of TLR4, such as the release of pro-inflammatory factors, contribute to the adverse effects of morphine. To address this, in this experiment I used the selective TLR4 antagonist LPS-RS (Lipopolysaccharide from *Rhodobacter sphaeroides*; InvivoGen, San Diego, CA, USA). To test whether inhibition of the TLR4 signaling cascade blocks the adverse effects of morphine following SCI, subjects were pretreated with intrathecal LPS-RS (0, 20, or 40  $\mu\text{g}$  dissolved in endotoxin-free water). Thirty minutes later, half of the subjects in each dose group were treated with 90  $\mu\text{g}$  of morphine and the remaining subjects were treated with vehicle (0.09% saline). This 3 X 2 experimental design (6 groups) used a total of 60 rats ( $n=10$ ).

#### *Effects of LPS-RS on the analgesic efficacy of morphine*

Sensory function was evaluated using the tail-flick test to establish baseline thermal reactivity thresholds. There were no significant differences between the groups prior to treatment ( $F(5, 50) = 1.24$ ,  $p > 0.05$ ; data not shown). A two-factor ANOVA on post-treatment scores, however, revealed significant main effects of morphine ( $F(1, 50) = 2390.94$ ,  $p < 0.001$ ) and LPS-RS treatment ( $F(2, 50) = 8.64$ ,  $p < 0.05$ ), and a significant interaction ( $F(2, 50) = 8.64$ ,  $p < 0.05$ ). As shown in Figure 30, across all



*Figure 30: Effects of LPS-RS on the analgesic efficacy of morphine.* The analgesic efficacy of morphine following LPS-RS pre-treatment is depicted for the test of thermal reactivity. All groups that received morphine had significantly increased tail-flick latencies relative to vehicle-treated controls. Analgesia was also higher for subjects treated with the highest dose of LPS-RS alone. Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  or post-hoc tests; #  $p < 0.05$  for planned comparisons; *M* = significant main effect of morphine.

LPS-RS doses, subjects that received morphine showed significantly increased tail-flick latencies relative to their saline-treated counterparts. Moreover, subjects pre-treated with 40 µg of LPS-RS alone showed increased analgesia in comparison to those in the 0 or 20 µg groups. The test was automatically terminated at 8 seconds to prevent tissue injury.

#### *Effects of LPS-RS on locomotor recovery*

Locomotor recovery was monitored for 21 days post-injury (Figure 31). Locomotor scores collected before treatment on day 1 did not differ significantly across groups ( $F(5, 50) = 0.25$ ,  $p > 0.05$ ). Mean converted BBB scores ( $\pm$  S.E.M.) on day 1

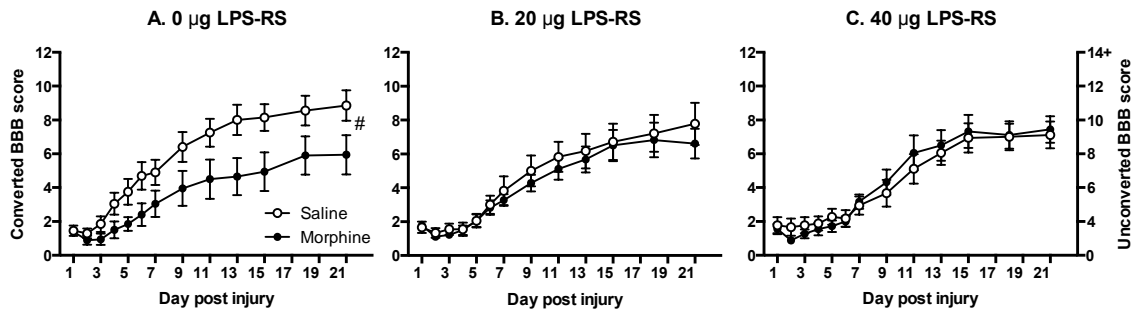


Figure 31: Effects of LPS-RS on locomotor recovery. LPS-RS pretreatment blocked the adverse effects of morphine on long-term recovery of locomotor function. Morphine undermined recovery of locomotor function in the absence of LPS-RS (A). Pretreatment with 20 (B) or 40 (C)  $\mu$ g LPS-RS, however, prevented the morphine-induced attenuation of locomotor recovery. Results shown as Mean  $\pm$  S.E.M. #  $p < 0.05$  for planned comparisons.

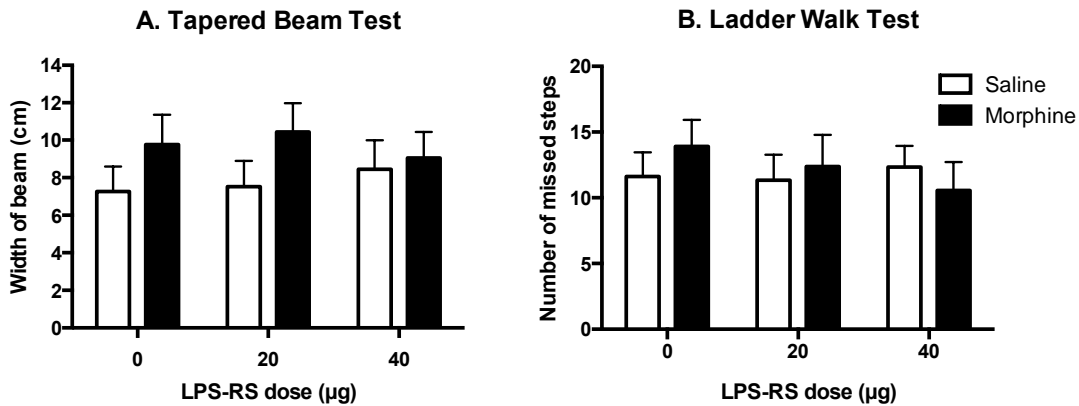


Figure 32: Effects of LPS-RS on beam and ladder walk performance. There were no significant effects of morphine or LPS-RS treatment on tapered beam (A) or ladder walk (B) performance 21-days post-injury. Results shown as Mean  $\pm$  S.E.M.

ranged from  $1.40 \pm 0.23$  to  $1.78 \pm 0.47$ . A mixed-design ANCOVA using day 1 scores as a covariate did not uncover significant main effects of morphine ( $F(1, 49) = 3.02, p > 0.05$ ) or LPS-RS treatment ( $F(2, 49) = 0.94, p > 0.05$ ), and no significant interaction ( $F$

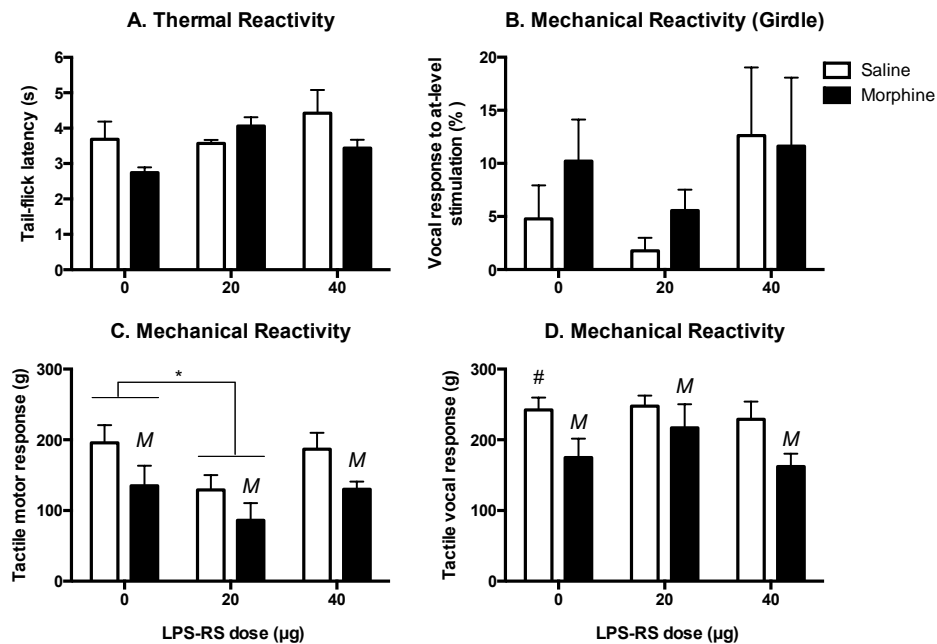
(2, 49) = 2.54,  $p > 0.05$ ). Replicating our previous studies (Hook et al., 2009; Hook et al., 2011), planned comparisons revealed that control subjects (0  $\mu$ g of LPS-RS) treated with morphine had significantly reduced locomotor recovery when compared to their saline-treated counterparts ( $p < 0.05$ ). In contrast, there were no differences between morphine- and saline-treated subjects receiving 20  $\mu$ g ( $p > 0.05$ ) or 40  $\mu$ g ( $p > 0.05$ ) of LPS-RS.

Motor recovery was further evaluated at the end of the 21-day recovery period using the tapered beam and ladder walk tests (Figure 32). Using a two-factor ANOVA, I did not find significant main effects of morphine ( $F(1, 49) = 2.49$ ,  $p > 0.05$ ), LPS-RS ( $F(2, 49) = 0.04$ ,  $p > 0.05$ ), or an interaction between treatments ( $F(2, 49) = 0.31$ ,  $p > 0.05$ ), on tapered beam performance. Similarly, I did not find any significant effects on ladder walk performance [morphine ( $F(1, 49) = 0.09$ ,  $p > 0.05$ ), LPS-RS ( $F(2, 49) = 0.21$ ,  $p > 0.05$ ), morphine X LPS-RS interaction ( $F(2, 49) = 0.50$ ,  $p > 0.05$ )].

#### *Effects of LPS-RS on sensory reactivity*

Long-term effects on sensory function were assessed at the end of the 21-day recovery period using the tail-flick, von Frey, and girdle tests (Figure 33). In the tail-flick test, mean latencies ( $\pm$  S.E.M.) ranged from  $2.74 \pm 0.15$  to  $4.42 \pm 0.66$  seconds. Statistical analyses showed no significant main effect of morphine ( $F(1, 49) = 2.17$ ,  $p > 0.05$ ), LPS-RS ( $F(2, 49) = 1.93$ ,  $p > 0.05$ ), or an interaction between the two ( $F(2, 49) = 2.10$ ,  $p > 0.05$ ), on thermal reactivity after day 21. In the von Frey test of mechanical reactivity, a two-factor ANOVA revealed significant main effects of morphine ( $F(1, 49) = 7.07$ ,  $p < 0.05$ ) and LPS-RS ( $F(2, 49) = 3.20$ ,  $p < 0.05$ ) on motor responses, but no

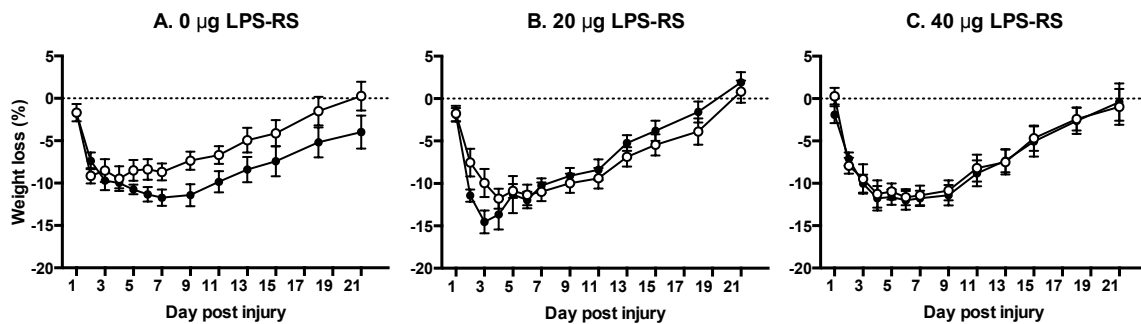
significant interaction ( $F(2, 49) = 0.07, p > 0.05$ ). On vocal responses, analyses revealed a significant main effect of morphine ( $F(1, 49) = 7.51, p < 0.05$ ), but no effect of LPS-RS ( $F(2, 49) = 1.09, p < 0.05$ ) or an interaction between morphine and LPS-RS ( $F(2, 49) = 0.35, p < 0.05$ ). Overall, morphine administration at all doses appeared to lower the threshold for mechanical reactivity at the spinal and supraspinal level. Lastly, I did not find any significant effects of morphine ( $F(1, 50) = 0.54, p > 0.05$ ), LPS-RS ( $F(2, 50) = 1.67, p > 0.05$ ), or their interaction ( $F(2, 50) = 0.27, p > 0.05$ ), in the girdle test of at-level allodynia.



**Figure 33: Effects of LPS-RS on sensory reactivity.** There were no significant main effects of morphine or LPS-RS treatment on thermal hyperalgesia (A) or at-level allodynia (B) tested at 21-days post-injury. However, both treatments affected motor responses to mechanical stimulation of the hindpaws (C). Morphine treatment also lowered the threshold for vocal responses in the von Frey test (D). Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests; #  $p < 0.05$  for planned comparisons; M = significant main effect of morphine.

### *Effects of LPS-RS on general health*

Weight immediately before injury did not differ across groups ( $F(5, 50) = 1.90$ ,  $p > 0.05$ ; data not shown). Commensurate with all other experiments, all groups lost weight following SCI (Figure 34). However, I did not find any significant effects of morphine ( $F(1, 50) = 1.08$ ,  $p > 0.05$ ), LPS-RS ( $F(2, 50) = 0.11$ ,  $p > 0.05$ ), or an interaction ( $F(2, 50) = 0.57$ ,  $p > 0.05$ ), on weight change across recovery.



*Figure 34: Effects of LPS-RS on weight change across recovery.* Weight loss was unaffected by morphine or LPS-RS treatment. Results shown as Mean  $\pm$  S.E.M.

Mortality, autophagia, spastic hypertonia, and bladder control were also monitored throughout the 21-day recovery period. Mortality was not affected by drug treatment. Only 1 subject died in the entire experiment (originally in the 0 µg LPS-RS + morphine group; subject was replaced). Similarly, only 1 case of mild autophagia was observed (in the 0 µg LPS-RS + saline group), but this subject did not need to be replaced. Three subjects displayed signs of spastic hypertonia in this experiment (1 each in the 0 µg LPS-RS + saline and 0 µg LPS-RS + morphine groups, and 1 in the 20 µg LPS-RS + morphine group). Lastly, I found a significant main effect of LPS-RS ( $F(2,$

50) = 3.25,  $p > 0.05$ ) on recovery of bladder control, but no effect of morphine ( $F(1, 50) = 0.16$ ,  $p > 0.05$ ) or any interaction ( $F(2, 50) = 0.37$ ,  $p > 0.05$ ; data not shown). On average, subjects treated with 40  $\mu\text{g}$  of LPS-RS showed slower recovery of bladder control than their vehicle-treated counterparts (20.78 days versus 18.70 days, respectively).

#### *Non-classic opioid receptor activation*

##### **Exp. 4A Sufficiency of non-classic opioid receptor activation**

In this experiment I used the unnatural [ + ]- enantiomer of morphine to assess the contribution of non-classic opioid receptor activation to the morphine-induced attenuation of function after SCI. To test whether activation of non-classic opioid receptors in the spinal cord following injury is sufficient to reproduce the adverse effects of morphine, [ + ]- morphine was dissolved in 10 N hydrochloric acid and titrated with 1N sodium hydroxide to pH 5–6, then diluted to the intended concentration with 0.9 % saline. The solution was then administered via an intrathecal route, as described in Chapter II General Methodology. A single dose of [ + ]- morphine was tested (0.32  $\mu\text{mol}$ ), equivalent to the effective dose of morphine seen in previous studies, along with a vehicle control (0  $\mu\text{mol}$ ). With an  $n=8$  per group, this experimental design used a total of 16 rats.

#### *Analgesic efficacy of [ + ]- morphine*

Sensory function was evaluated using the tail-flick test to establish baseline thermal reactivity thresholds. Prior to treatment, tail-flick scores ( $\pm$  S.E.M.) were  $3.98 \pm$

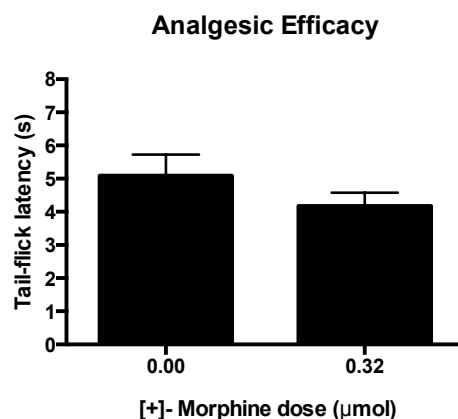


Figure 35: Analgesic efficacy of [+] morphine. Administration of [+] morphine did not produce analgesia. Results shown as Mean  $\pm$  S.E.M.

0.17 s and  $3.68 \pm 0.19$  s for the vehicle and [+] morphine groups, respectively. Statistical analyses showed that there were no significant differences between the groups at this time point ( $F(1, 14) = 1.23$ ,  $p > 0.05$ ; data not shown). As expected, selective activation of non-classic opioid receptors, using the unnatural [+] enantiomer of morphine, did not result in analgesia ( $F(1, 14) = 1.30$ ,  $p > 0.05$ ; Figure 35). Since [+] morphine failed to produce analgesia at the high dose of 0.32  $\mu\text{mol}$ , lower doses were not tested.

#### *Effects of [+] morphine on locomotor recovery*

Administration of the unnatural [+] enantiomer of morphine did not have any significant effects on recovery of locomotor function (Figure 36), despite administering a dose equivalent to the natural [-] enantiomer of morphine previously shown to undermine recovery (Hook et al., 2009; Hook et al., 2011). Mean converted BBB scores ( $\pm$  S.E.M.) collected before treatment on day 1 were  $1.88 \pm 0.33$  for vehicle-treated



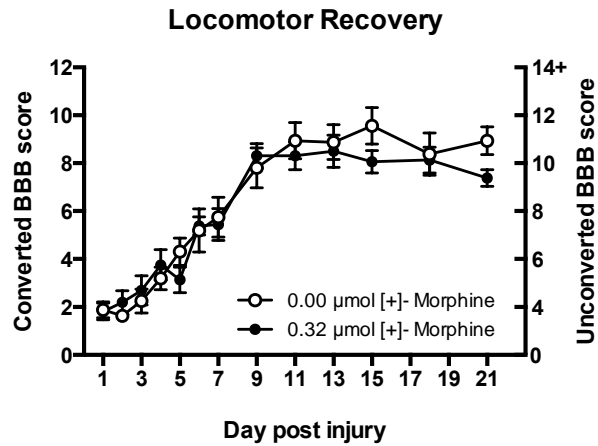


Figure 36: Effects of [±]- morphine on locomotor recovery. Subjects were monitored for 21 days following a moderate contusion SCI. Intrathecal administration of [±]-morphine, on day 1 post-injury, did not affect locomotor recovery as assessed using the BBB scale. Results shown as Mean  $\pm$  S.E.M.

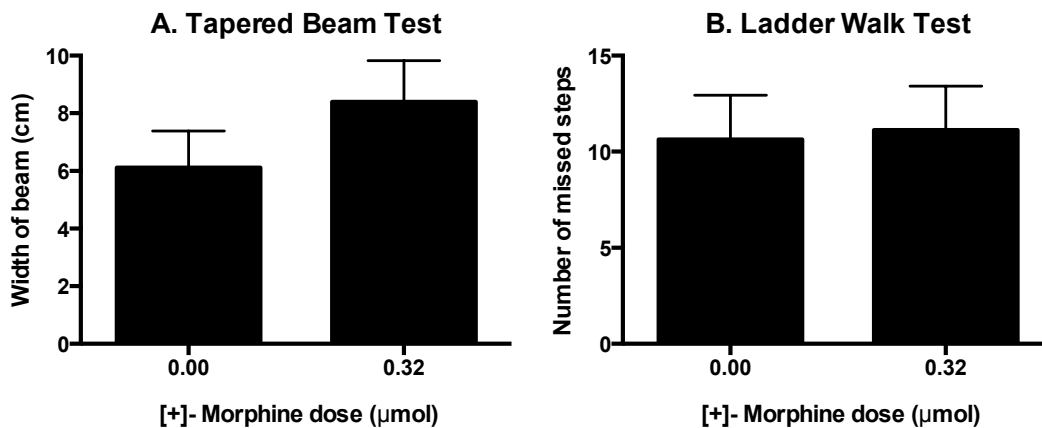


Figure 37: Effects of [±]- morphine on beam and ladder walk performance. At the end of the 21-day recovery period, locomotor recovery was further assessed using the tapered beam (A) and ladder walk (B) tests. [±]- Morphine had no effect on performance of these tests. Results shown as Mean  $\pm$  S.E.M.

controls and  $1.81 \pm 0.35$  for  $[+]$ - morphine-treated subjects, and did not differ statistically ( $F(1, 14) = 0.02, p > 0.05$ ). Using day 1 scores as a covariate, a mixed-design ANOVA revealed no differences in locomotor recovery between the groups across the 21 days post-injury ( $F(1, 14) = 0.20, p > 0.05$ ). Further, as shown in Figure 37, there were no significant differences between treatment groups after day 21 on either the beam ( $F(1, 14) = 1.25, p > 0.05$ ) or ladder ( $F(1, 14) = 0.02, p > 0.05$ ) tests.

#### *Effects of $[+]$ - morphine on sensory reactivity*

Sensory function was assessed at the end of the 21-day recovery period using the tail-flick, von Frey, and girdle tests (Figure 38). As observed with locomotor recovery, treatment with the unnatural morphine enantiomer did not affect sensory reactivity at day 21 post-injury. The groups did not show any differences in thermal reactivity assessed with the tail-flick test ( $F(1, 14) = 0.86, p > 0.05$ ). Similarly, no differences were observed for motor ( $F(1, 14) = 0.28, p > 0.05$ ) or vocal reactivity ( $F(1, 14) = 1.18, p > 0.05$ ) to a mechanical stimulus applied to the hindpaws. Finally, administration of  $[+]$ - morphine did not affect at-level allodynia assessed with the girdle test ( $F(1, 14) = 1.16, p > 0.05$ ).

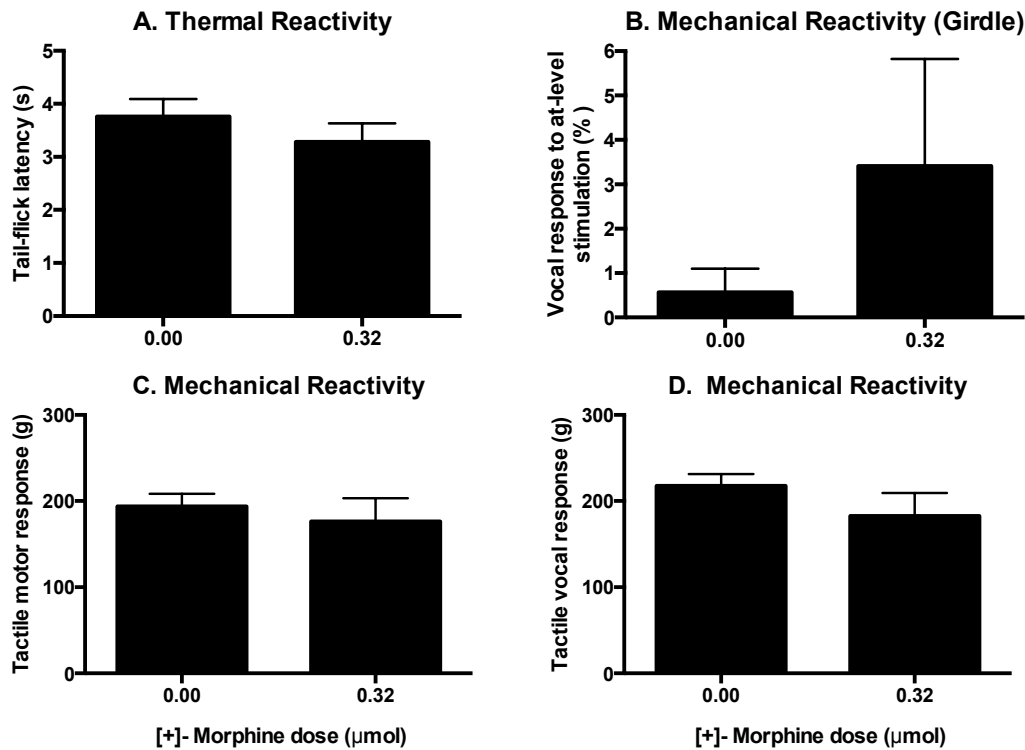


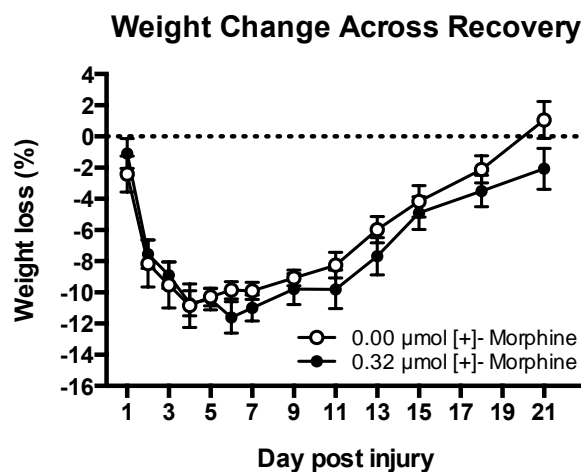
Figure 38: Effects of [±]- morphine on sensory reactivity. At the end of the 21-day recovery period, sensory reactivity was assessed using the tail-flick (A), girdle (B), and von Frey (C, D) tests. [±]- Morphine administration did not affect long-term pain reactivity. Results shown as Mean  $\pm$  S.E.M.

#### Effects of [±]- morphine on general health

Weight was monitored as an index of general health. Weight immediately before injury differed across groups ( $F(1, 14) = 11.54, p < 0.05$ ; data not shown). Mean weight immediately before injury ( $\pm$  S.E.M.) was  $372.50 \pm 3.04$  g for subjects treated with [±]-morphine and  $347.13 \pm 6.29$  for controls. To assess changes in weight across recovery taking into account initial differences, the daily % weight loss was calculated by subtracting the starting weight (weight at the day of surgery) from daily weight. A

repeated-measures ANOVA on daily % weight loss values showed that there were no significant differences between the groups ( $F(1, 14) = 0.40, p > 0.05$ ; Figure 39).

In addition to weight, mortalities, autophagia, spastic hypertonia, and recovery of bladder control were also recorded to assess general health. I did not observe any significant effects of drug treatment on mortality (a single subject died in the vehicle group). None of the subjects recovered bladder control, and no spastic hypertonia was recorded throughout the duration of the experiment. Administration of  $[+]-$  morphine appeared to exacerbate autophagia, with 37.5% of  $[+]-$  morphine subjects demonstrating signs of self-harm, compared to 0% of vehicle-treated controls. This effect approached, but did not reach statistical significance ( $\chi^2 = 3.69, p = 0.055$ ).



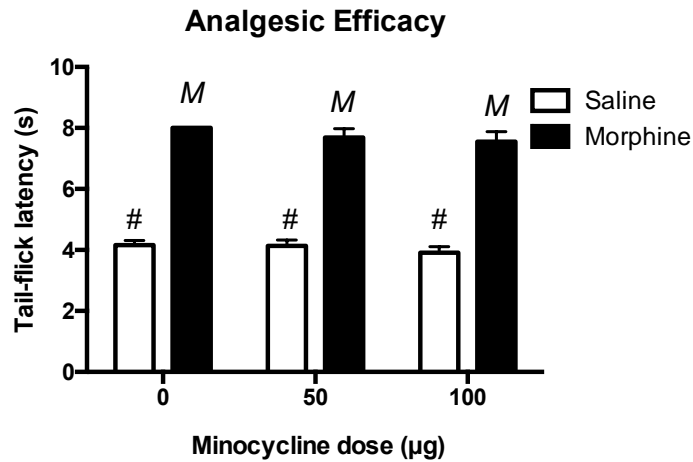
*Figure 39: Effects of  $[+]-$  morphine on weight change across recovery.* This graph depicts the daily % weight loss across the 21-day recovery period, calculated by subtracting starting weight from daily weight.  $[+]-$  morphine did not affect weight change, or other signs of general health, following SCI. Results shown as Mean  $\pm$  S.E.M.

#### **Exp. 4B Necessity of non-classic opioid receptor activation**

Since treatment with LPS-RS appeared to decrease the adverse effects of morphine, I hypothesized that the attenuation of function results from opioid-mediated increases in the innate inflammatory response following SCI. I posited that morphine increases glial activation, increasing the release of pro-inflammatory factors, and leading to neurotoxicity. To test whether glial inhibition prevents the adverse effects of morphine following SCI, subjects were pretreated with intrathecal minocycline (0, 50, or 100  $\mu\text{g}$  dissolved in distilled water). Fifteen minutes later, half of the subjects in each dose were treated with 90  $\mu\text{g}$  of morphine and the remaining subjects were treated with vehicle (0.09% saline). This 3 X 2 experimental design (6 groups) used a total of 60 rats ( $n=10$ ).

##### *Effects of minocycline on the analgesic efficacy of morphine*

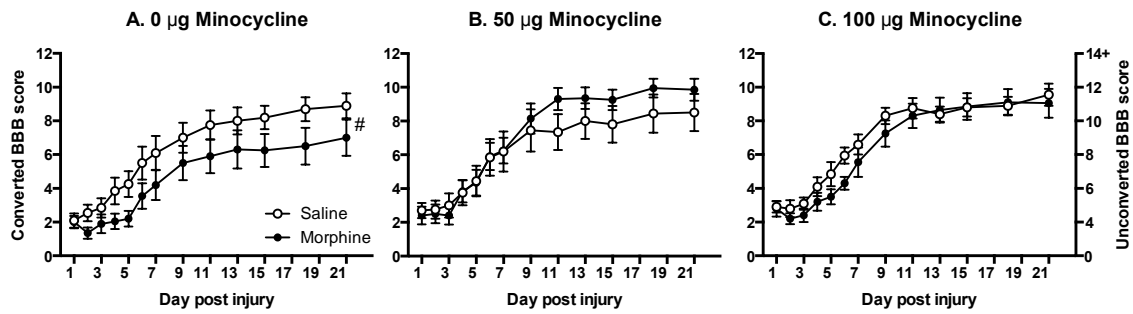
Sensory function was evaluated using the tail-flick test to establish baseline thermal reactivity thresholds. There were no significant differences between the groups prior to treatment ( $F(5, 54) = 1.58, p > 0.05$ ; data not shown). A two-factor ANOVA on post-treatment scores, however, revealed a significant main effect of morphine ( $F(1, 54) = 372.38, p < 0.001$ ), but no effect of minocycline treatment ( $F(2, 54) = 1.10, p > 0.05$ ) and no interaction ( $F(2, 54) = 0.21, p > 0.05$ ). As shown in Figure 40, across all doses, subjects that received morphine, irrespective of minocycline treatment, showed significantly increased tail-flick latencies relative to their saline-treated counterparts ( $p < 0.05$ ). The test was automatically terminated at 8 seconds to prevent tissue injury.



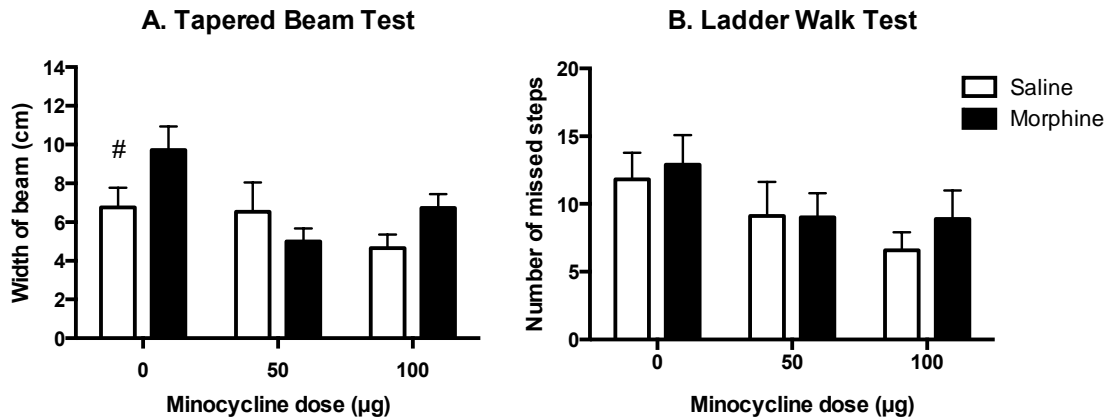
*Figure 40: Effects of minocycline on the analgesic efficacy of morphine.* The analgesic efficacy of morphine challenged with minocycline pre-treatment is depicted for the test of thermal reactivity. At all doses (0, 50, and 100 µg), subjects that received morphine had significantly increased tail-flick latencies relative to saline controls. Results shown as Mean  $\pm$  S.E.M. #  $p < 0.05$  for planned comparisons;  $M$  = significant main effect of morphine.

#### *Effects of minocycline on locomotor recovery*

Locomotor recovery was monitored for 21 days post-injury (Figure 41). Locomotor scores collected before treatment on day 1 did not differ significantly across groups ( $F(5, 54) = 0.70, p > 0.05$ ). Mean converted BBB scores ( $\pm$  S.E.M.) on day 1 ranged from  $2.50 \pm 0.50$  to  $3.70 \pm 0.64$ . A mixed-design ANCOVA using day 1 scores as a covariate did not uncover significant main effects of morphine ( $F(1, 53) = 0.86, p > 0.05$ ) or minocycline treatment ( $F(2, 53) = 0.55, p > 0.05$ ). Their interaction, however, reached significance ( $F(2, 53) = 3.26, p < 0.05$ ). Replicating our previous studies (Hook et al., 2009; Hook et al., 2011), planned comparisons revealed that control subjects (0 µg of minocycline) treated with morphine showed significantly reduced locomotor recovery



**Figure 41: Effects of minocycline on locomotor recovery.** Morphine significantly undermined locomotor function following SCI in subjects not treated with minocycline (A). However, minocycline pre-treatment at a 50  $\mu\text{g}$  (B) or 100  $\mu\text{g}$  dose (C) effectively blocked the adverse effects of morphine. Results shown as Mean  $\pm$  S.E.M. #  $p < 0.05$  for planned comparisons.



**Figure 42: Effects of minocycline on beam and ladder walk performance.** At the end of the 21-day recovery period, locomotor recovery was further assessed using the tapered beam (A) and ladder walk (B) tests. Morphine administration significantly undermined tapered beam performance in the absence of minocycline. No effects of morphine or minocycline were observed in ladder walk performance. Results shown as Mean  $\pm$  S.E.M. #  $p < 0.05$  for planned comparisons.

when compared to their saline-treated counterparts ( $p < 0.05$ ). In contrast, there were no differences between morphine- and saline-treated subjects receiving 50  $\mu\text{g}$  ( $p > 0.05$ ) or 100  $\mu\text{g}$  ( $p > 0.05$ ) of minocycline.

Motor recovery was further evaluated at the end of the 21-day recovery period using the tapered beam and ladder walk tests. A two-factor ANOVA uncovered a significant main effect of minocycline ( $F(2, 54) = 3.66, p < 0.05$ ) on the tapered beam test, but no effect of morphine ( $F(1, 54) = 1.77, p > 0.05$ ) or interaction ( $F(2, 54) = 2.45, p > 0.05$ ). As shown in Figure 42, there was a tendency for improved performance on the beam with minocycline administration. Additionally, morphine-treated subjects that did not receive minocycline showed the worst performance of all groups (unable to travel beyond an average width of 9.73 cm) and significantly differed from their saline-treated counterparts ( $p < 0.05$ ). I did not find any significant effects of morphine ( $F(1, 54) = 0.41, p > 0.05$ ) or minocycline ( $F(2, 54) = 2.51, p > 0.05$ ) treatment on ladder walk performance, and no interaction ( $F(2, 54) = 0.16, p > 0.05$ ).

#### *Effects of minocycline on sensory reactivity*

Long-term effects on sensory function were assessed at the end of the 21-day recovery period using the tail-flick, von Frey, and girdle tests. In the tail-flick test, mean latencies ( $\pm$  S.E.M.) ranged from  $3.74 \pm 0.25$  to  $4.70 \pm 0.42$  seconds. Statistical analyses revealed a significant main effect of minocycline treatment on thermal reactivity after day 21 ( $F(2, 54) = 3.41, p < 0.05$ ), but no effect of morphine ( $F(1, 54) = 0.004, p > 0.05$ ) or interaction ( $F(2, 54) = 0.11, p > 0.05$ ). As shown in Figure 43, controls (0  $\mu\text{g}$



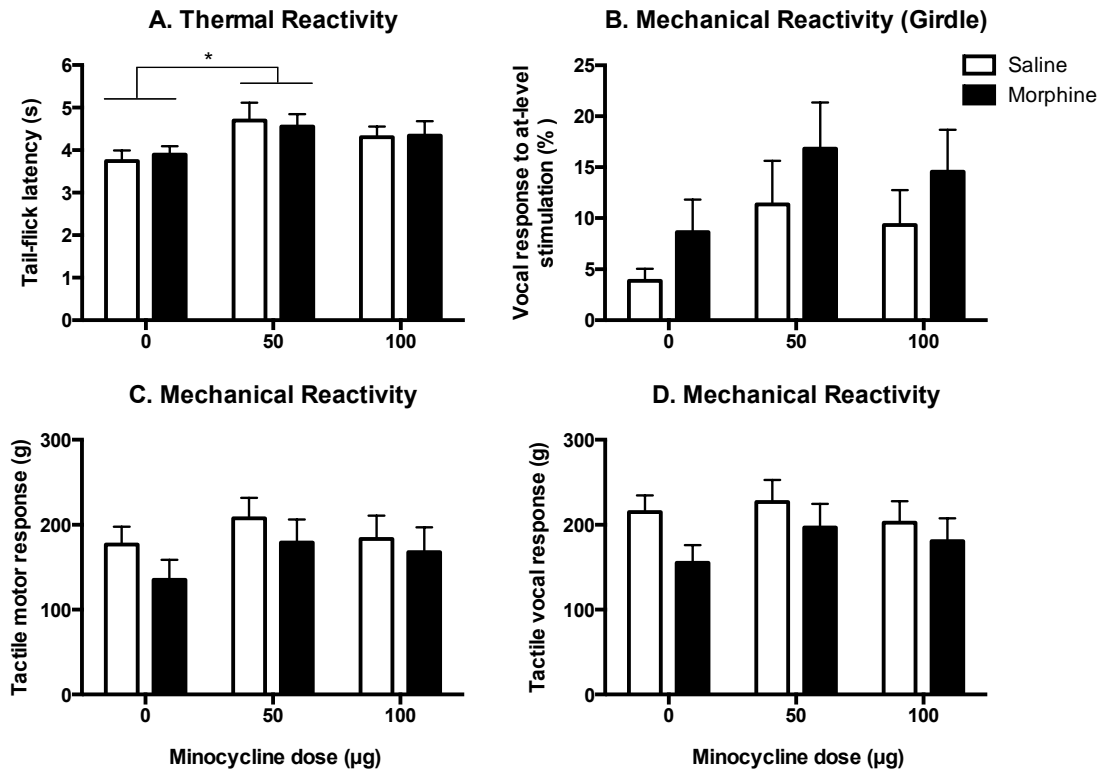


Figure 43: Effects of minocycline on sensory reactivity. Minocycline treatment decreased the development of thermal hyperalgesia (A), however, no effects of morphine or minocycline were observed on at-level allodynia (B) or mechanical reactivity of the hindpaws (C, D). Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests.

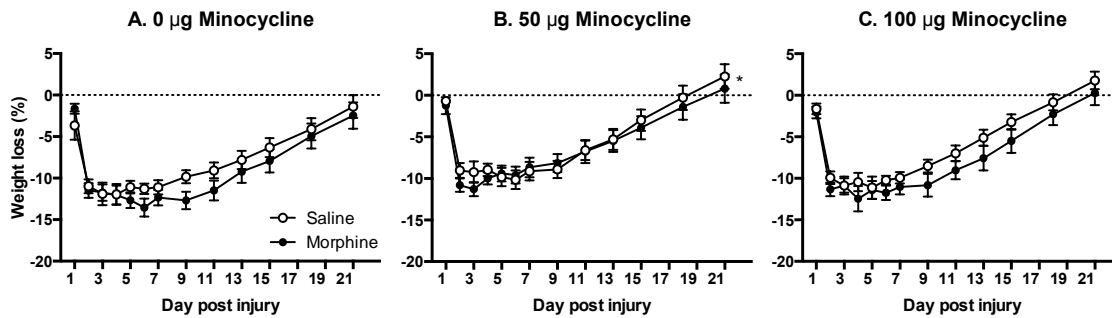
minocycline) showed lower thresholds than minocycline-treated subjects, regardless of morphine administration.

In the von Frey test of mechanical reactivity, although morphine-treated subjects showed lower motor thresholds than saline-treated animals, I did not find any significant effects of morphine ( $F(1, 52) = 1.67, p > 0.05$ ) or minocycline ( $F(2, 52) = 0.94, p > 0.05$ ), and no interaction ( $F(2, 52) = 0.12, p > 0.05$ ), on motor responses. On vocal responses to tactile stimulation, a main effect of morphine approached, but did not reach,

statistical significance ( $F(1, 52) = 3.07, p = 0.08$ ). Furthermore, no effect of minocycline ( $F(2, 52) = 0.56, p > 0.05$ ) or interaction ( $F(2, 52) = 0.31, p > 0.05$ ) was observed. Similarly, I did not find any significant effects of morphine ( $F(1, 54) = 2.72, p > 0.05$ ) or minocycline ( $F(2, 54) = 2.24, p > 0.05$ ), and no interaction ( $F(2, 54) = 0.004, p > 0.05$ ), on the girdle test of at-level allodynia.

#### *Effects of minocycline on general health*

Weight, monitored as an index of general health, did not differ across groups immediately before injury ( $F(5, 51) = 0.69, p > 0.05$ ; data not shown). To assess changes in weight across recovery, the daily % weight loss was calculated by subtracting the starting weight (weight at the day of surgery) from current weight. These scores were analyzed using a repeated-measures ANOVA, which revealed a significant main effect of minocycline ( $F(2, 51) = 5.17, p < 0.05$ ), but no effect of morphine ( $F(1, 51) = 2.08, p > 0.05$ ) and no interaction ( $F(2, 51) = 0.14, p > 0.05$ ). As shown in Figure 44, all groups lost weight following SCI. Treatment with minocycline, however, attenuated weight loss irrespective of morphine treatment. In fact, by the end of the recovery period, every group that received minocycline returned to their pre-injury weight, but this was not the case for controls (0  $\mu$ g minocycline).



*Figure 44: Effects of minocycline on weight change across recovery.* The average daily % weight loss is depicted for the 21-day recovery period. Treatment with minocycline attenuated weight loss following SCI, irrespective of morphine treatment. Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests (in comparison to 0  $\mu\text{g}$  controls).

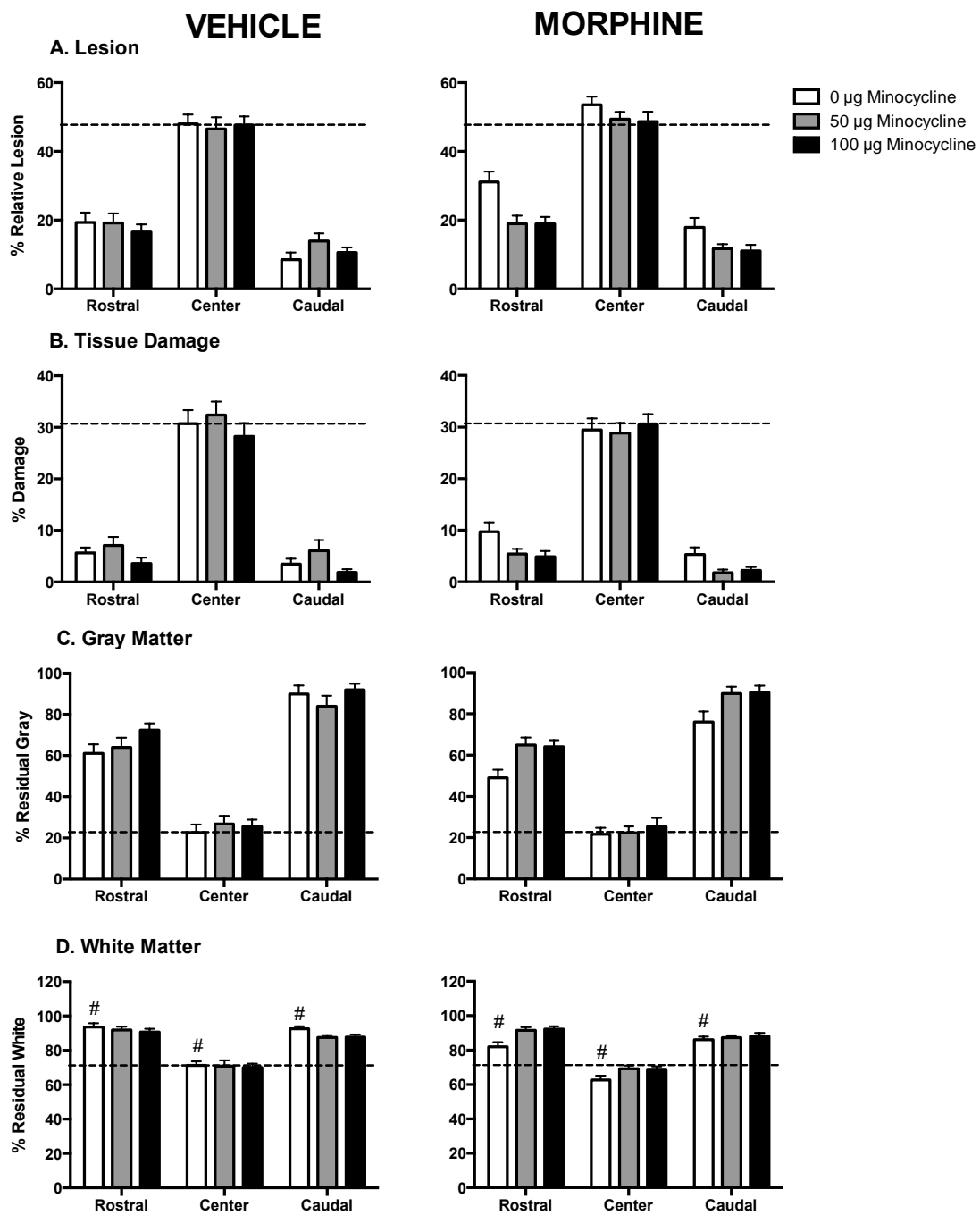
I also recorded mortality, autophagia, spastic hypertonia, and bladder control throughout the 21-day recovery period. There were no mortalities, and very few instances of autophagia and spastic hypertonia. Overall, there were only two cases of autophagia (both treated with 50  $\mu\text{g}$  minocycline, 1 each in the morphine and saline groups), and four subjects showing signs of spastic hypertonia (all treated with morphine, 2 each pretreated with 0 or 100  $\mu\text{g}$  minocycline). Lastly, neither morphine ( $F(5, 51) = 0.69, p > 0.05$ ) nor minocycline ( $F(5, 51) = 0.69, p > 0.05$ ) affected recovery of bladder control (data not shown).

#### *Effects of minocycline on tissue sparing at the site of injury*

To assess whether the effects of minocycline on locomotor function were the result of increased neuroprotection, I quantified the amount of tissue sparing in the spinal cord at the end of the recovery period (Figure 45). Spinal tissue was subdivided into rostral (2400 – 1200  $\mu\text{m}$ ), center (600 – -600  $\mu\text{m}$ ), and caudal (-1200 – -2400  $\mu\text{m}$ )

segments. Four measures were analyzed: relative lesion size (damage + missing tissue), tissue damage, residual gray matter, and residual white matter. Across the rostral-caudal extent of the spinal cord, there were no significant main effects on any of these four measures (for all,  $p > 0.05$ ). Planned comparisons between saline and morphine-treated subjects, however, showed differences in residual white matter across the rostral-caudal extent of the lesion, only in subjects that were not pretreated with minocycline. It appears that minocycline treatment protected residual white matter in morphine-treated subjects commensurate to that of their saline-treated counterparts.

*Figure 45: Effects of minocycline on tissue sparing at the site of injury.* At the end of the experiment, subjects were perfused and tissue was collected for histological assessment. There were no significant effects of minocycline on lesion size (A), damage (B), residual gray matter (C), or residual white matter (D). However, differences in white matter sparing were found at every segment between saline and morphine-treated subjects without minocycline. Results shown as Mean  $\pm$  S.E.M. # $p < 0.05$  for planned comparisons.



## Discussion

In these experiments I evaluated the contribution of non-classic opioid receptor activation to the morphine-induced attenuation of function following SCI. First, I assessed the role of TLR4 using the selective agonist LPS. At a high dose of 100  $\mu$ g, LPS significantly decreased the analgesic efficacy of morphine in sham subjects, but did not result in adverse consequences in contused subjects (Experiment 3.A). To further explore the role of TLR4 in morphine's effects following SCI, I challenged morphine treatment with the highly-selective antagonist LPS-RS (Experiment 3.B). Pre-treatment with LPS-RS, at 20 and 40  $\mu$ g, did not disrupt morphine's analgesic efficacy. However, at both doses, LPS-RS appeared to reduce the adverse effects of morphine on recovery of function. This suggests that it is possible that I did not see an effect of TLR4 activation with LPS due to the immune response innate to SCI (ceiling effect).

Since selective activation of TLR4 did not replicate morphine's adverse effects, next I evaluated the contribution of overall non-classic opioid receptor activation using the unnatural enantiomer of morphine (Experiment 4.A). As expected, I found that [+-] morphine does not produce analgesia, confirming that the classic opioid receptors were not activated by the treatment. At a dose commensurate with an effective dose of morphine, I found that [+-] morphine does not have any adverse effects on recovery following SCI. However, as observed with LPS-RS, reducing glial activation with minocycline blocked the adverse effects of morphine on locomotor recovery, prevented the development of thermal hyperalgesia and increased tissue sparing at the injury site.

The data presented here support previous findings from our laboratory (Hook et al., 2007; Hook et al., 2009; Hook et al., 2011). In our rodent SCI model, we have shown that morphine administration significantly increases the levels of pro-inflammatory cytokines as early as 30 minutes post-injection (Hook et al., 2011). Notably, we have also shown that blocking the interleukin-1 (IL-1) receptor prior to morphine treatment prevents the attenuation of locomotor function and reduces symptoms of at-level neurophatic pain (Hook et al., 2011). In the current studies I showed that blocking TLR4 activation with LPS-RS, or using minocycline to inhibit glia at the site of injury, also prevents morphine-induced attenuation of function. Altogether, our findings suggest that opioids interact with the immune system to undermine recovery following SCI. The mechanisms leading to these adverse effects, however, remain unclear and should be further investigated.

While few studies have looked at the immunomodulatory effects of opioids following SCI, opioid-immune interactions have been extensively documented in other injury models (Hutchinson et al., 2007; Hutchinson et al., 2011; Watkins et al., 2007a; Watkins et al., 2007b; Watkins et al., 2009). Research in pre-clinical pain models, for instance, have characterized a TLR4-mediated mechanism through which morphine contributes to the initiation and maintenance of paradoxical pain (Grace et al., 2016; Hutchinson et al., 2011; Hutchinson et al., 2010b). Briefly, this mechanism posits that morphine forms a complex with TLR4 and MD2, which then activates intracellular signaling cascades via the recruitment of adaptor proteins. These proteins engage downstream signaling pathways, such as the phosphatidylinositol 3-kinase (PI3K)/Akt

pathway, involved in cell motility, survival, and apoptosis, or the NF- $\kappa$ B and MAPK pathways, responsible for the production and release of pro-inflammatory cytokines and chemokines (Hutchinson et al., 2011).

Surprisingly, in the studies presented here, selective activation of TLR4 using LPS and of other non-classic opioid receptors with [+] morphine did not replicate the adverse effects of morphine previously observed in our SCI model (Hook et al., 2007; Hook et al., 2009; Hook et al., 2011). It is possible that the effect of these agonists was masked by the immune response innate to SCI. In comparison to the nerve injury models, which have provided the majority of evidence supporting an opioid-TLR4 relationship, SCI results in a more robust pro-inflammatory response. Indeed, within hours, SCI leads to an extensive upregulation of TLRs and their downstream regulators in the injured spinal cord (Kigerl et al., 2007). Specifically, studies have shown increased mRNA levels for TLR1, 2, 4, 5, and 7, as well as the adaptor protein MyD88, and the transcription factor NF- $\kappa$ B. To further contrast the role of TLR signaling in nerve injury and SCI models, genetic knockout of TLR2 and TLR4 in a mouse model of SCI resulted in increased inflammation, increased gliosis, and decreased recovery of locomotor function (Kigerl et al., 2007). This suggests that following SCI, TLR activation may approach a ceiling effect, masking any other effects of LPS or [+] morphine. I posit that opioids engage additional molecular pathways- for example, via KOR signaling- to augment the inflammatory response and undermine recovery of function.



The data presented here also suggest that, although activation of TLR4 and other non-classic opioid receptors is not sufficient to replicate morphine's effects, the immune system plays a critical role in the attenuation of recovery. Pharmacological modulation of the immune response appears to prevent morphine's adverse consequences without compromising the innate immune response necessary for recovery of function. Indeed, our findings suggest that anti-inflammatories should be further investigated as adjuvants to improve the safety and efficacy of opioids in the clinical setting. In animal models of nerve injury, for example, treatment with glial inhibitors like minocycline and pentoxifylline significantly potentiates morphine analgesia (Mika et al., 2007) and decreases tolerance (Cui et al., 2008; Mika et al., 2009). Blocking glial activation also has other benefits, such as attenuating the development of allodynia and hyperalgesia after injury to the nervous system (Chang & Waxman, 2010; Ledeboer et al., 2005; Mika et al., 2007). Further corroborating our results, research in other laboratories also suggest that glial inhibitors can be powerful tools for neuroprotection in models of traumatic brain and spinal cord injury (Mejia et al., 2001; Stirling et al., 2004). Notably, minocycline and other FDA-approved anti-inflammatories are currently under investigation in a variety of clinical trials as treatments for neurodegenerative conditions like Huntington disease and Parkinson disease (Kim & Suh, 2009; Plane et al., 2010). Overall, these drugs appear to be promising clinical tools, and warrant further investigation as adjuvants to opioid regimens for spinally-injured patients.

## CHAPTER V

### CELLULAR MECHANISMS: EFFECTS OF MORPHINE ADMINISTRATION AT THE SITE OF INJURY

#### **Introduction**

The analyses of classic and non-classic receptor binding, presented in Chapters 3 and 4, suggest that both KOR signaling and glial activation contribute to the adverse effects of morphine administration following SCI. While the literature strongly supports a role of TLR signaling in the immunomodulatory effects of opioids, LPS and [+] morphine failed to replicate the adverse effects of morphine consistently shown in our SCI model. Altogether, the findings presented here point to a classically-mediated mechanism of action underlying morphine's unwanted side effects. I posit that morphine undermines recovery following SCI by binding to KORs on microglia/macrophages, and biasing these cells toward a pro-inflammatory profile. Indeed, evidence suggests that following trauma to the nervous system, responding microglia/macrophages adopt either an M1 phenotype, which is pro-inflammatory and neurotoxic, or an M2 phenotype, which is anti-inflammatory and promotes repair (Kigerl et al., 2009). In the already highly inflammatory context of a SCI, KOR signaling may induce a positive-feedback loop through which aberrant glial activation results in secondary damage to residual tissue.

Supporting an interaction between the classic opioid receptors and immune system, studies have demonstrated that cells involved in host defense, including

peripheral blood mononuclear cells (T-cells and B-cells), granulocytes, and peritoneal macrophages, also express classic opioid receptor mRNA (Bidlack et al., 2006; Chuang et al., 1995; Sharp, 2006). Even within the nervous system, opioid receptor expression is not restricted to neurons. Astrocytes, oligodendrocytes, and microglia have been shown to express classic opioid receptors (Belcheva et al., 2005; Bruchas et al., 2006; Chao et al., 1996; Chao et al., 1997; Eriksson et al., 1993; Gurwell et al., 1996; Hauser et al., 1996; McLennan et al., 2008; Ruzicka et al., 1995; Stiene-Martin & Hauser, 1991; Stiene-Martin et al., 2001; Stiene-Martin et al., 1993; Stiene-Martin et al., 1998; Xu et al., 2007). Importantly, research shows that some of these cells can exhibit altered opioid receptor expression in response to inflammation or injury.

Evidence suggests that cytokines and chemokines present in the cellular environment can trigger changes in opioid receptor expression on immune cells and glia. For example, IL-1 $\beta$  can regulate opioid peptide production and opioid receptor expression in astrocyte-enriched cultures, but its effects differ depending on the opioid receptor subtype and brain regions under investigation (Ruzicka & Akil, 1997; Ruzicka et al., 1996). In these studies, treatment with IL-1 $\beta$  for 24 hours increased the level of MOR mRNA by 55-75% in cultures from rat striatum, cerebellum, and hippocampus, but not in those derived from the cortex or hypothalamus. Conversely, the same treatment reduced KOR mRNA in all cultures, but had no effect on DOR mRNA. Simultaneous treatment with IL-1 $\alpha$  and IL-1 $\beta$  has also been shown to increase MOR expression in neural microvascular endothelial cell cultures (Vidal et al., 1998). These

data suggest that opioid-immune interactions may be dramatically altered by the pathophysiological context in which they are applied.

I hypothesize that in the acute phase of SCI morphine binds to KORs expressed on glial cells, leading to increased activation, and subsequently increased cell death at the site of lesion. In order to test this, in the following experiments I used immunohistochemistry and flow cytometry to characterize the cellular environment at the site of lesion, with and without morphine treatment. Our results show that at 48 hours following injury, morphine administration does not significantly change the number of neurons or astrocytes present. However, morphine treatment results in a robust increase in the number of microglia/macrophages regardless of injury. Injury alone also significantly increases the number of microglia/macrophages with MOR and KOR expression. Furthermore, as opposed to their saline-treated counterparts, contused subjects that receive morphine had significantly more KOR+ microglia at the site of injury. This supports our pharmacological studies, and underscores the need to further investigate the use of opioids in the context of trauma.

## **Methods and results**

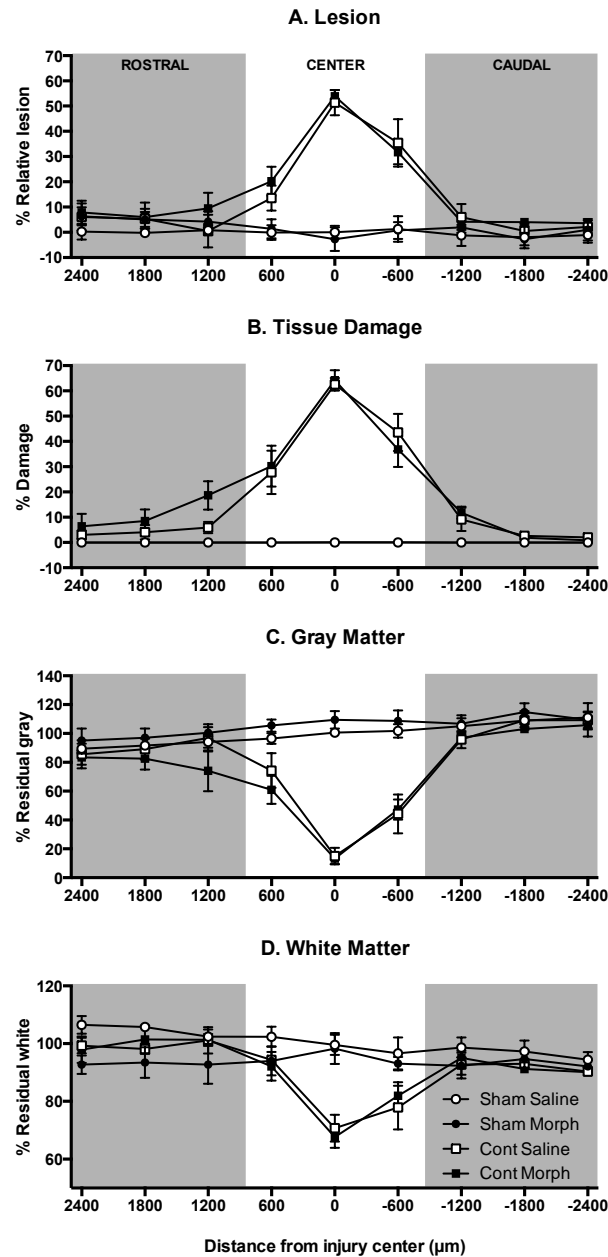
### *Cellular effects assessed with immunohistochemistry*

In this experiment I used immunohistochemistry (IHC) to assess the effects of morphine administration at the site of a spinal cord injury. I hypothesized that sub-acute morphine treatment alters spinal cellular populations that play a key role in secondary injury, leading to attenuation of function in the chronic stages of recovery. To test this, I quantified neurons, astrocytes, and macrophages at the injury epicenter. Briefly,

subjects received a sham or contusion surgery. Twenty-four hours after injury, morphine (90 µg) or vehicle (filtered 0.9% saline) were administered via an intrathecal catheter. Twenty-four hours after drug treatment (48 hr post-injury), subjects were humanely euthanized, and tissue was collected for immunohistochemical analyses as described in Chapter II General Methodology. With an n=4 per group, this 2 (surgery) X 2 (drug) experimental design used a total of 16 subjects.

#### **Exp. 5A Histological assessment of lesion size in the acute phase of contusion injury**

First, I assessed the lesion size using histology (Figure 46). Spinal tissue was subdivided into rostral (2400 – 1200 µm), center (600 – -600 µm), and caudal (-1200 – -2400 µm) segments. Four measures were analyzed: relative lesion size (damage + missing tissue), tissue damage, residual gray matter, and residual white matter. For relative lesion size, no significant effects were observed in the rostral and caudal segments (for all,  $p > 0.05$ ). However, in the center segment, I found a significant main effect of surgery ( $F(1, 12) = 68.52, p < 0.05$ ), but no effect of drug treatment ( $F(1, 12) = 0.02, p > 0.05$ ) or surgery X drug treatment interaction ( $F(1, 12) = 0.09, p > 0.05$ ). Surgery also significantly affected the amount of damage at the rostral ( $F(1, 12) = 6.60, p < 0.05$ ), center ( $F(1, 12) = 186.75, p < 0.05$ ), and caudal ( $F(1, 12) = 19.54, p < 0.05$ ) segments of the spinal cord. However, no effects of drug treatment or interactions on damage to the spinal cord were observed throughout the rostral-caudal extent of the lesion (for all,  $p > 0.05$ ) at this early time point. Not surprisingly, contused subjects had



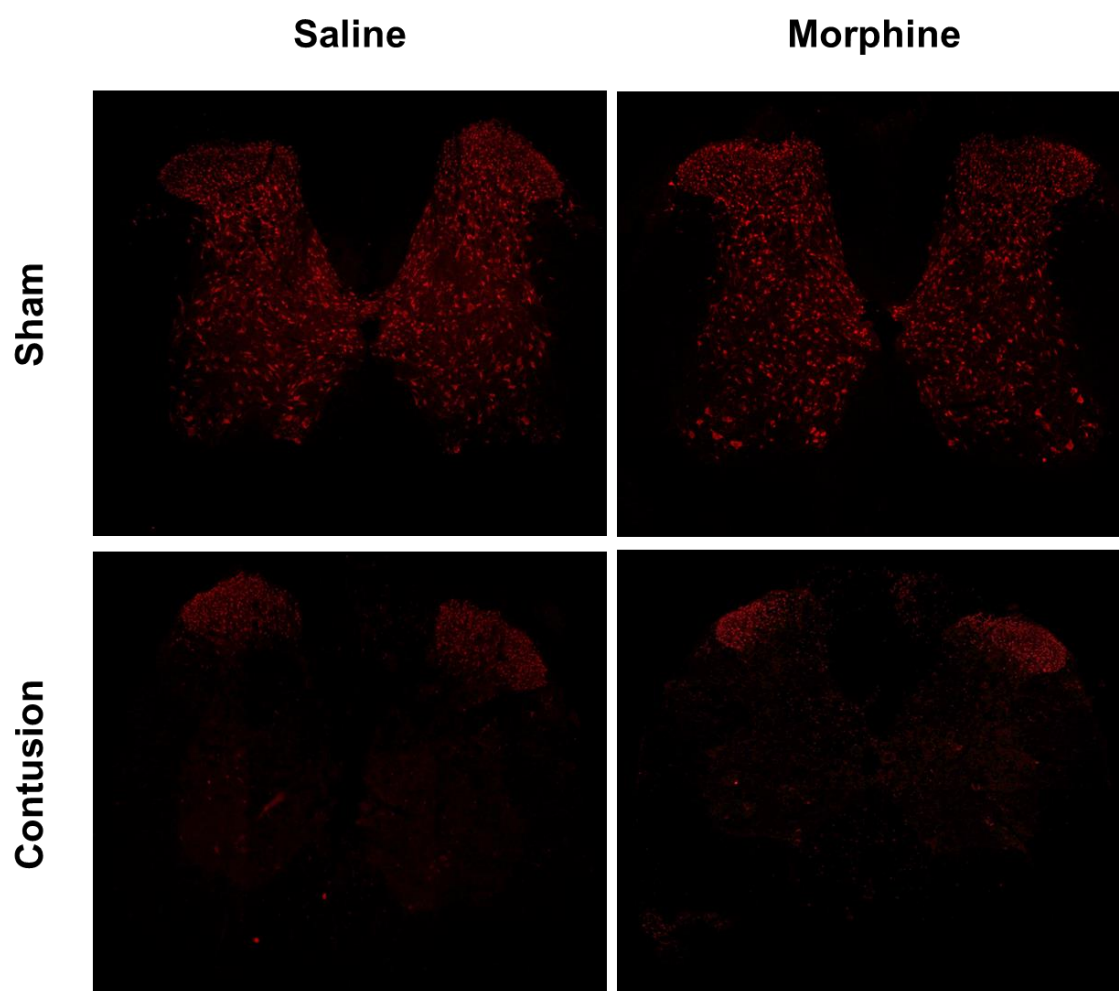
*Figure 46: Tissue sparing at the site of injury.* Two days post-SCI, subjects were perfused and tissue was collected for histological assessment. There was a significant main effect of surgery at the center spinal segment for relative lesion (A), residual gray matter (C), and residual white matter (D). Surgery also affected damage throughout the rostral-caudal spinal cord, with no effect of drug treatment or interaction (B). Results shown as Mean  $\pm$  S.E.M.

a bigger lesion and more damage than their sham counterparts, regardless of drug treatment.

For residual gray matter, there were no significant effects of surgery or drug treatment in the rostral and caudal segments (for all,  $p > 0.05$ ). However, in the center segment, I found a significant main effect of surgery ( $F(1, 12) = 129.11, p < 0.05$ ), but no effect of drug treatment ( $F(1, 12) = 0.16, p > 0.05$ ) or surgery x drug treatment interaction ( $F(1, 12) = 1.30, p > 0.05$ ). Similarly, for residual white matter, no significant effects were found in the rostral and caudal segments (for all,  $p > 0.05$ ), but in the center segment, there was a significant main effect of surgery ( $F(1, 12) = 14.28, p < 0.05$ ), with no effect of drug treatment ( $F(1, 12) = 0.30, p > 0.05$ ) or significant interaction ( $F(1, 12) = 0.21, p > 0.05$ ). Overall, although contused subjects lost more gray and white matter than their sham counterparts, there was no significant effect of morphine treatment on residual tissue at this early time point.

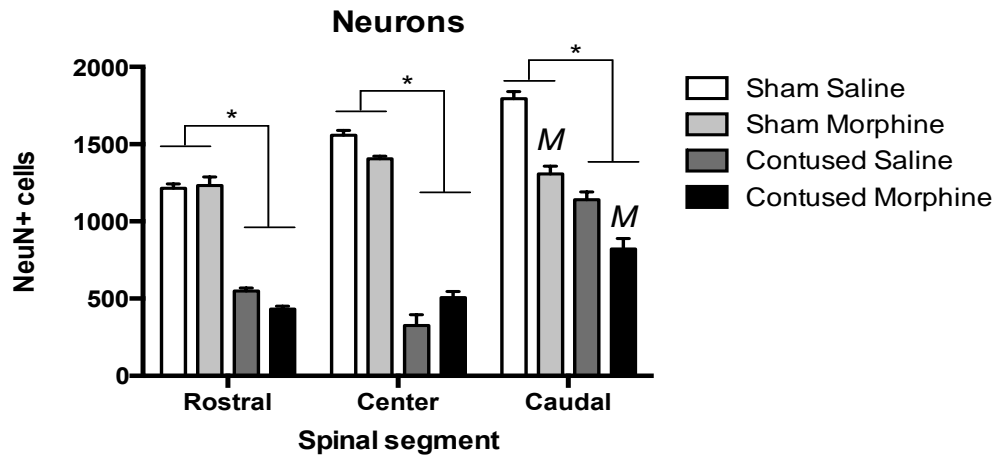
#### **Exp. 5B Neurons, astrocytes, and microglia/macrophages at the lesion epicenter**

Following the histological assessment of the lesion area, I used immunohistochemistry (IHC) to investigate whether morphine treatment alters key cellular populations following SCI. Spinal tissue was prepared for IHC and images were analyzed using ImageJ software as described in Chapter II General Methodology. First I quantified the number of neurons (NeuN+ cells; Figure 47) present across the rostral-caudal extent of the lesion. In the rostral segment (1200 – 2400  $\mu\text{m}$ ), a mixed-design ANOVA uncovered a significant main effect of surgery ( $F(1, 12) = 16.19, p < 0.05$ ), but



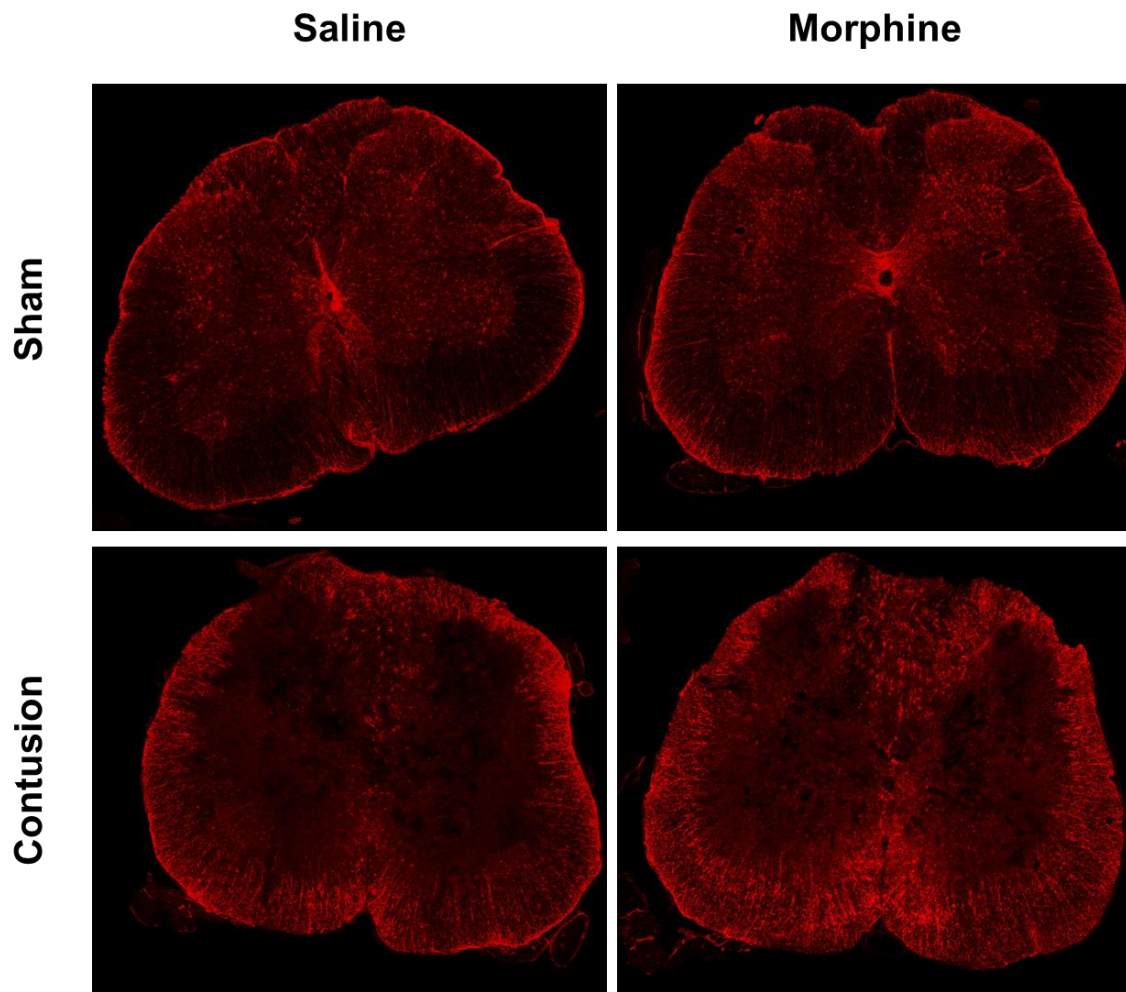
*Figure 47: Visualizing neurons using immunohistochemistry.* Neurons in the spinal cord were visualized using NeuN antibodies. These sections were taken from the center of the injury at 4X magnification.



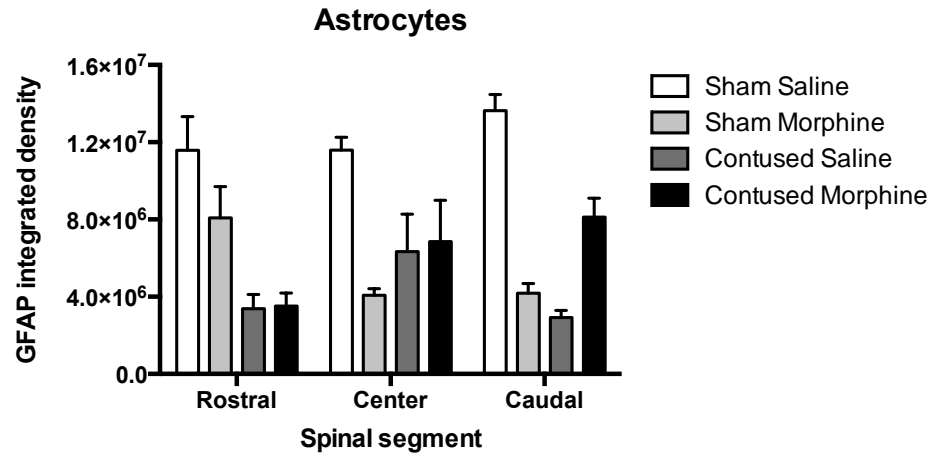


*Figure 48: Quantification of neurons at the injury epicenter.* Quantification of NeuN+ cells at the rostral, center, and caudal spinal segments are shown in this graph. There was a significant main effect of surgery across all segments, with contused subjects losing more neurons than sham subjects. Morphine treatment also significantly decreased neuron number, but only in the caudal segment. There were no significant interactions between surgery and drug treatment at any spinal level. Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  or post-hoc tests; *M* = significant main effect of morphine.

no effect of drug treatment ( $F(1, 12) = 0.08, p > 0.05$ ) or interaction ( $F(1, 12) = 0.14, p > 0.05$ ; Figure 48). At 48 hrs post injury, there were significantly more neurons present in sham tissue than contused tissue, regardless of drug treatment. Similarly, in the center segment (-600 – 600  $\mu\text{m}$ ), there was an effect of surgery ( $F(1, 12) = 56.17, p < 0.05$ ), but no effect of drug treatment ( $F(1, 12) = 0.01, p > 0.05$ ) or interaction ( $F(1, 12) = 1.36, p > 0.05$ ). Caudally (-2400 – -1200  $\mu\text{m}$ ), however, there were significant main effects of both surgery ( $F(1, 12) = 9.14, p < 0.05$ ), and drug treatment ( $F(1, 12) = 4.57, p < 0.05$ ), although the two did not interact ( $F(1, 12) = 0.19, p > 0.05$ ). At this level, contused subjects and morphine-treated subjects had less neurons than their sham and saline-treated counterparts, respectively.



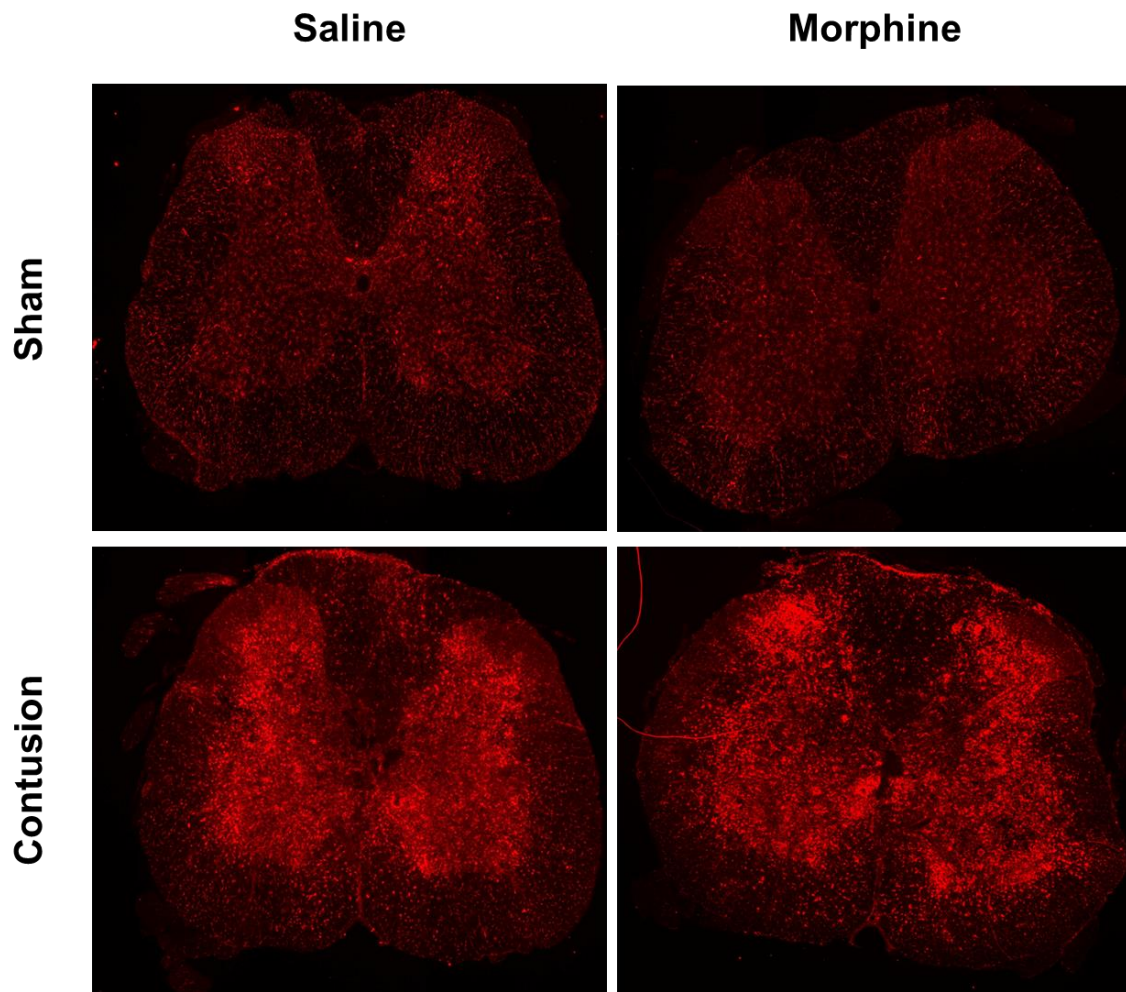
*Figure 49: Visualizing astrocytes using immunohistochemistry. Astrocytes in the spinal cord were visualized using GFAP antibodies. These sections were taken from the center of the injury at 4X magnification.*



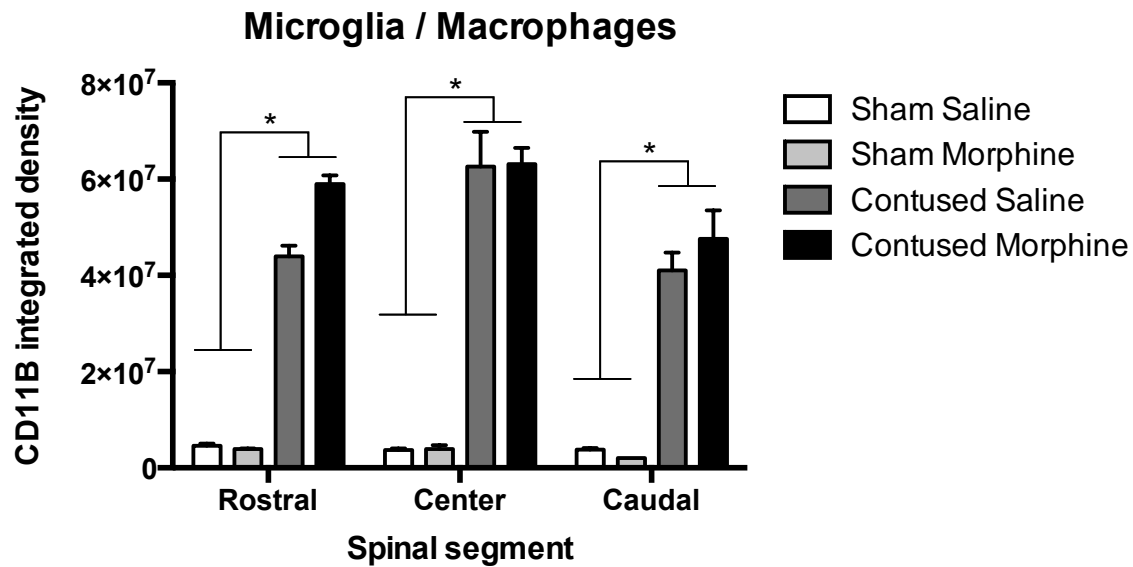
*Figure 50: Quantification of astrocytes at the injury epicenter.* Quantification of GFAP expression at the rostral, center, and caudal spinal segments is shown in this graph. There were no significant effects of surgery or drug treatment at any level of the spinal cord. Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  or post-hoc tests;  $M$  = significant main effect of morphine.

Next I assessed astrogliosis across the rostral-caudal extent of the lesion by quantifying the expression of GFAP (Figure 49). As previously described, I used mixed-design ANOVAs to assess the effects of surgery and morphine treatment in the rostral, center, and caudal spinal segments. In the rostral segment, there was no effect of drug treatment ( $F(1, 11) = 0.03, p > 0.05$ ) or interaction ( $F(1, 11) = 0.05, p > 0.05$ ), and a main effect of surgery approached but did not reach significance ( $F(1, 11) = 3.35, p = 0.09$ ; Figure 50). Similarly, I did not find any significant effects in the center or caudal segments (for all,  $p > 0.05$ ). The data suggest that neither surgery nor morphine significantly affect astrogliosis across the rostral-caudal extent of the lesion at 48 hour post-injury.

I also quantified CD11b (Ox42) expression, a marker of microglia and other macrophages, to assess inflammation across the rostral-caudal extent of the lesion (Figure 51). Mixed-design ANOVAs showed that in the rostral segment, there was a significant main effect of surgery ( $F(1, 12) = 43.49, p < 0.05$ ), but no effect of drug treatment ( $F(1, 12) = 0.99, p > 0.05$ ) or interaction ( $F(1, 12) = 1.20, p > 0.05$ ; Figure 52). Contused subjects showed a robust increase in CD11b expression compared with their sham counterparts. I also found similar results in the center [main effect of surgery ( $F(1, 12) = 46.52, p < 0.05$ ), no effect of drug treatment ( $F(1, 12) = 0.002, p > 0.05$ ) or interaction ( $F(1, 12) = 0.00, p > 0.05$ )] and caudal [main effect of surgery ( $F(1, 12) = 94.25, p < 0.05$ ), no effect of drug treatment ( $F(1, 12) = 0.32, p > 0.05$ ) or interaction ( $F(1, 12) = 0.93, p > 0.05$ )] segments. As expected, injury increases the presence and/or activation of immune cells across the rostral-caudal extent of the lesion.



*Figure 51: Visualizing microglia/macrophages using immunohistochemistry.* Microglia and macrophages in the spinal cord were visualized using CD11b antibodies. These sections were taken from the center of the injury at 4X magnification.



*Figure 52: Quantification of microglia / macrophages at the injury epicenter.* Quantification of CD11b (Ox42) expression at the rostral, center, and caudal spinal segments is shown in this graph. There was a significant main effect of surgery across all segments, with contused subjects showing higher CD11b expression than sham subjects. Morphine treatment did not significantly affect CD11b expression, and there were no significant surgery X drug treatment interactions at any spinal level. Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  or post-hoc tests;  $M$  = significant main effect of morphine.

#### *Changes in the inflammatory response assessed with flow cytometry*

In this experiment I used flow cytometry to further assess opioid-immune interactions following spinal cord injury. I decided to use flow cytometry because, it allows for the rapid and reliable characterization and quantification of individual cells. This would have been difficult to perform using immunohistochemistry due to the high density of immune cells in spinal cord sections following injury. Based on the protective effects of minocycline (Chapter 4), I hypothesized that morphine exacerbates the innate immune response following SCI, increasing the neurotoxic environment, and producing the attenuated recovery of locomotor function observed in our model. To test this, I

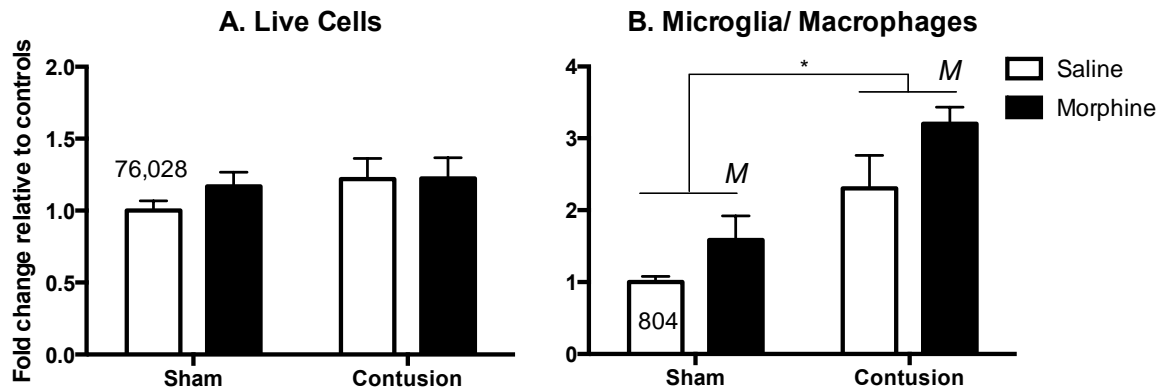
quantified immune cells (resident and infiltrating macrophages) at the injury epicenter and their expression of opioid receptors. As in the previous experiment, subjects received a sham or contusion surgery. Twenty-four hours after injury, morphine (90  $\mu$ g) or vehicle (filtered 0.9% saline) were administered via an intrathecal route. Twenty-four hours after drug treatment (48 hr post-injury), subjects were humanely euthanized, and tissue was collected for flow cytometry as described in Chapter II General Methodology. With an  $n=6$  per group, this 2 (surgery) X 2 (drug) design used a total of 24 subjects.

### **Exp. 6A Infiltrating macrophages and microglia**

To assess opioid-immune interactions that could lead to decreased recovery of function in our model, I began by characterizing the infiltrating and resident response following injury and drug treatment. First, I used forward and side scatter to select live cells in our heterogeneous samples. There were no significant differences in the number of total live cells across the groups (for all,  $p > 0.05$ ). Next, I used markers for CD11b and IBA1 to quantify the total number of microglia and macrophages present in our samples (Figure 53). As expected, I found a significant main effect of surgery ( $F(1, 20) = 18.76$ ,  $p < 0.05$ ) on the total number of microglia/macrophages, with increased numbers due to injury alone. Statistical analyses also showed that there was a significant main effect of drug treatment ( $F(1, 20) = 4.85$ ,  $p < 0.05$ ). Regardless of injury, morphine administration increased the total number of microglia/macrophages at the site of the lesion. Finally, although there was no significant interaction between surgery and drug treatment ( $F(1, 20) = 0.22$ ,  $p > 0.05$ ), their effects may be cumulative, since subjects that received morphine after a contusion had the highest levels of

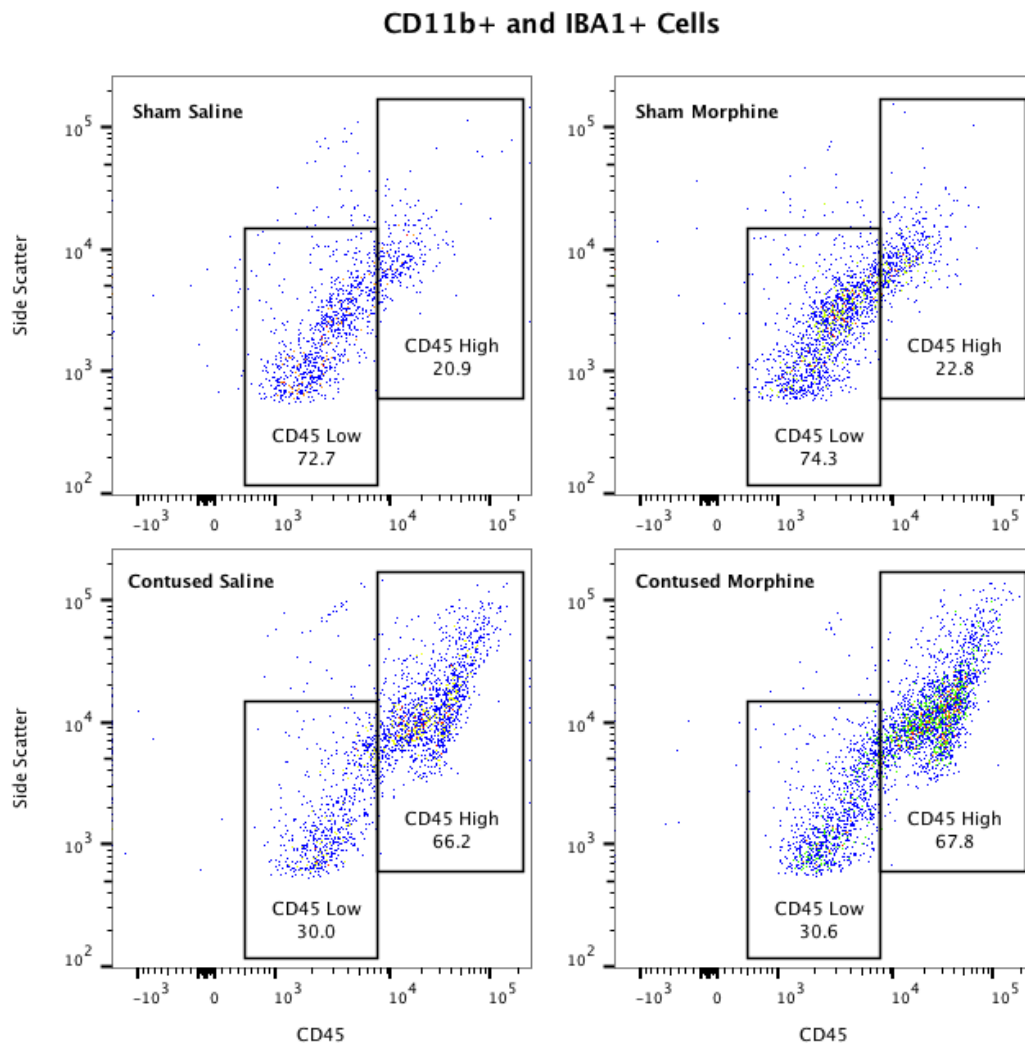
microglia/macrophages at the injury site.

To further evaluate the local and peripheral immune response, the cells identified as microglia/macrophages were further classified into infiltrating macrophages (high CD45 expression) or resident microglia (low CD45 expression) as shown in Figure 54. Not surprisingly, I found that injury significantly increased the number of infiltrating macrophages ( $F(1, 20) = 20.17, p < 0.05$ ; Figure 55). However, there was no significant main effect of drug treatment ( $F(1, 20) = 0.68, p > 0.05$ ) or interaction ( $F(1, 20) = 0.26, p > 0.05$ ) on infiltrating macrophages. Alternatively, drug treatment significantly increased the number of microglia present at the site of injury ( $F(1, 20) = 5.31, p < 0.05$ ), but there was no significant main effect of surgery ( $F(1, 20) = 1.00, p > 0.05$ ) or a surgery X drug treatment interaction ( $F(1, 20) = 0.01, p > 0.05$ ).

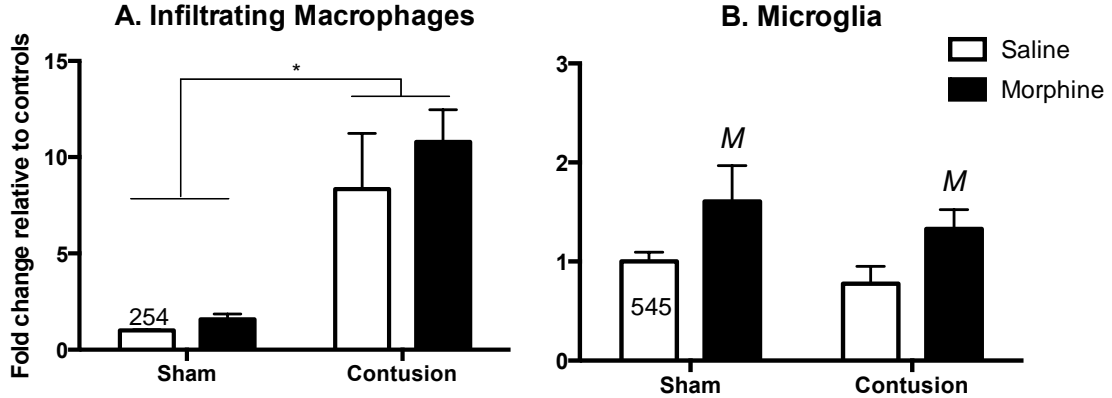


**Figure 53: Live cells and microglia / macrophages at the injury epicenter.** Microglia and macrophages were quantified using CD11b and IBA1 antibodies. Surgery alone and drug treatment alone significantly affected the total number of microglia/macrophages at the site of injury, with no significant interaction. Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests; *M* = significant main effect of morphine. Total number of cells in sham + saline group indicated in the first bar.



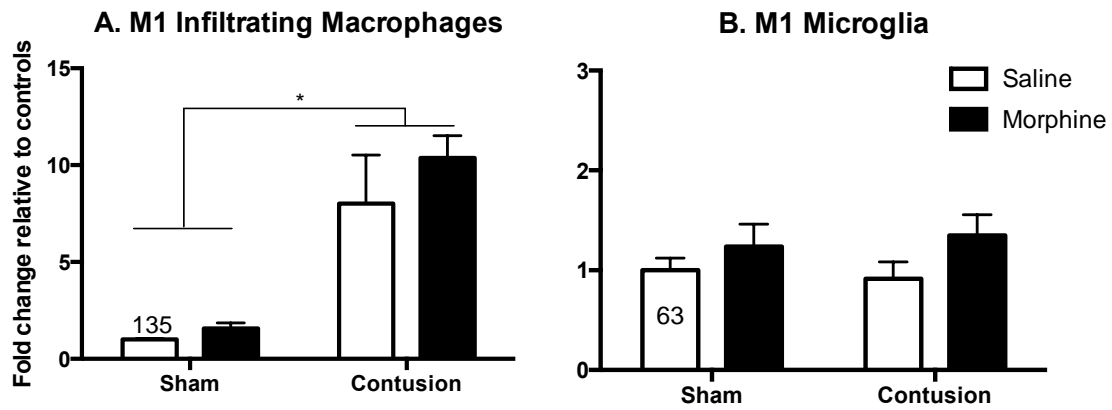


*Figure 54: Pseudocolor plot of macrophages and microglia at the injury epicenter. Cells positive for CD11b and IBA1 markers are displayed here using a pseudocolor plot (warmer colors indicate higher cell density). The cells were further subdivided according to CD45 expression, as shown by the boxes. The number in the boxes indicates the percentage of cells encompassed by the gate out of the total CD11b+ and IBA1+ parent population.*



**Figure 55: Infiltrating macrophages and microglia at the injury epicenter.** The local and peripheral immune response was characterized using CD45 expression. Injury increased the number of infiltrating macrophages with no effect of drug treatment (A). On the other hand, morphine increased the number of resident microglia with no effect of surgery (B). No significant interactions were observed. Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests; *M* = significant main effect of morphine. Total number of cells in sham + saline group indicated in the first bar.

To further characterize whether the infiltrating macrophages and resident microglia were also M1 polarized, I used markers for CD68 and CD86. Although not all previously identified infiltrating macrophages expressed CD68 and CD86, the overall effects remained (Figure 56). Injury significantly increased the number of M1 infiltrating macrophages ( $F(1, 20) = 27.39, p < 0.05$ ), with no effect of drug treatment ( $F(1, 20) = 0.93, p > 0.05$ ) or interaction ( $F(1, 20) = 0.35, p > 0.05$ ). On the other hand, there was no significant effect of surgery ( $F(1, 20) = 0.004, p > 0.05$ ), drug treatment ( $F(1, 20) = 2.73, p > 0.05$ ), or interaction ( $F(1, 20) = 0.24, p > 0.05$ ) on M1 microglia, although slight elevations after morphine treatment were observed.



*Figure 56: M1 polarization of macrophages and microglia at the injury epicenter.* M1 polarization was assessed using markers for CD68 and CD86. Injury increased the number of M1 infiltrating macrophages with no effect of drug treatment (A). No significant main effects were observed on M1 microglia (B). Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests. Total number of cells in sham + saline group indicated in the first bar.

### Exp. 6B MOR co-localization

I hypothesized that the adverse effects of opioids in our model could also be related to changes in opioid receptor expression following injury. To investigate this, I assessed overall MOR expression and its co-localization on immune cells following SCI and morphine treatment (Figure 57). As done previously, first I assessed the total number of live cells in the heterogeneous samples using forward and side scatter. There were no significant main effects of surgery ( $F(1, 20) = 1.55, p > 0.05$ ) or drug treatment ( $F(1, 20) = 3.07, p > 0.05$ ), but a planned comparisons test showed that there were more live cells in the sham-morphine versus the sham-saline group. Next, I quantified the total number of MOR-expressing cells at the injury center (this includes immune cells, as well

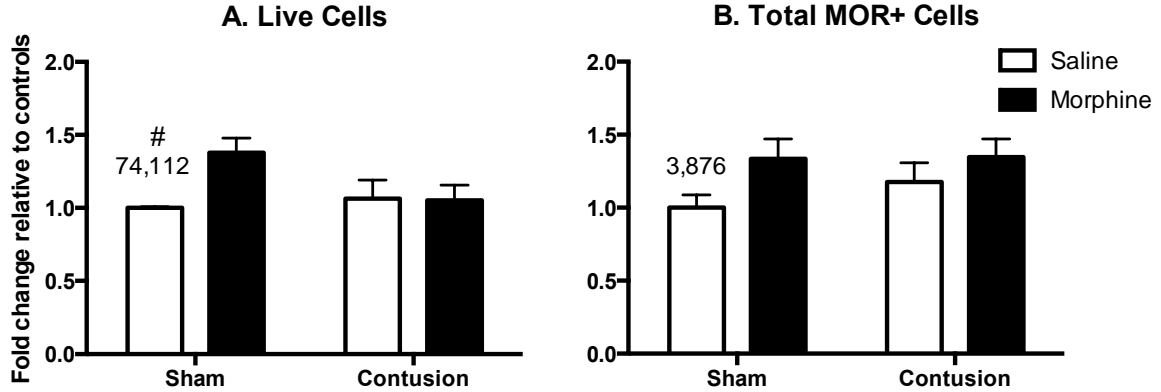
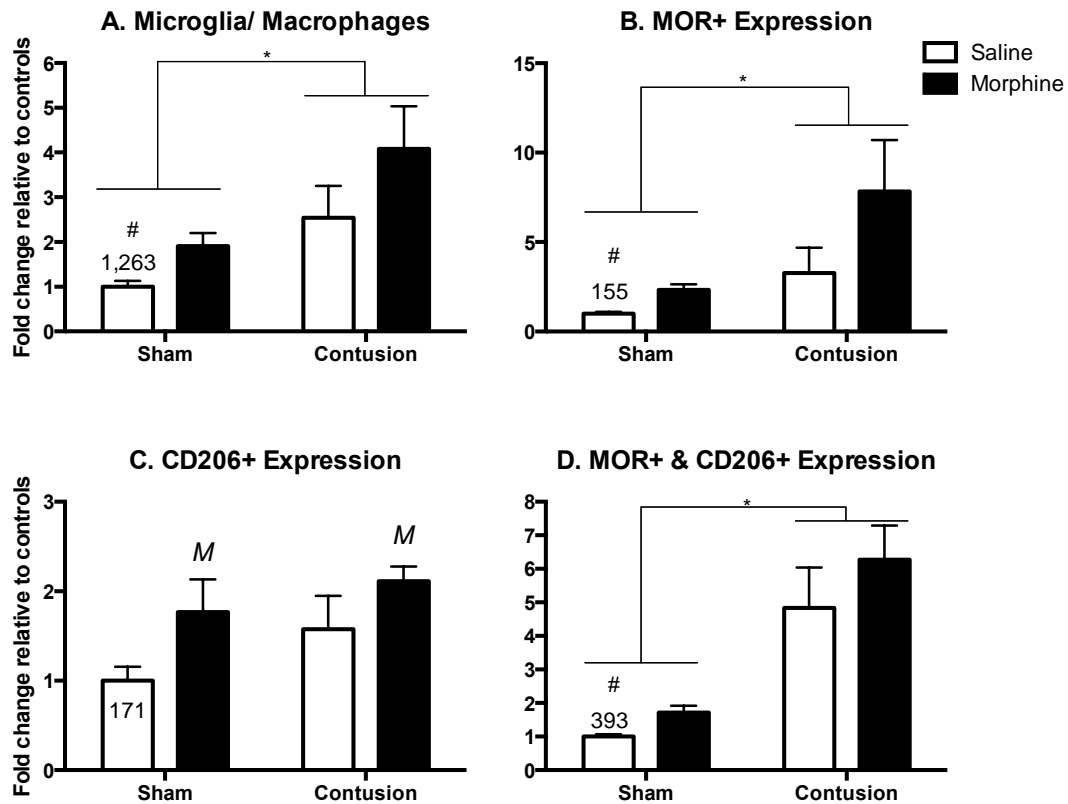


Figure 57: MOR+ cells at the injury epicenter. There were no significant main effects of surgery or drug treatment on total MOR+ cells at the site of injury. Total number of cells in sham + saline group indicated in the first bar.

as other cell types such as neurons and astrocytes). I did not find any significant effects of surgery ( $F(1, 20) = 0.49, p > 0.05$ ), drug treatment ( $F(1, 20) = 3.58, p > 0.05$ ), or an interaction ( $F(1, 20) = 0.38, p > 0.05$ ) on total MOR+ cells.

Although I did not find any effects on total MOR+ cells, I also wanted to explore MOR expression specifically on immune-competent cells. To do this, once again I identified microglia/macrophages using CD11b and IBA1 markers (Figure 58). Our results in this experiment paralleled those obtained in Exp. 6A. I found a significant main effect of surgery ( $F(1, 20) = 7.62, p < 0.05$ ) on the number of microglia/macrophages. Drug treatment approached, but did not reach significance ( $F(1, 20) = 3.30, p > 0.05$ ), and there was no significant interaction ( $F(1, 20) = 0.38, p > 0.05$ ). Next, I looked at microglia/macrophages that also expressed MOR and CD206. I found a significant main effect of surgery ( $F(1, 20) = 4.86, p < 0.05$ ) on the number of MOR+ microglia/macrophages, but no effect of drug treatment ( $F(1, 20) = 2.79, p >$



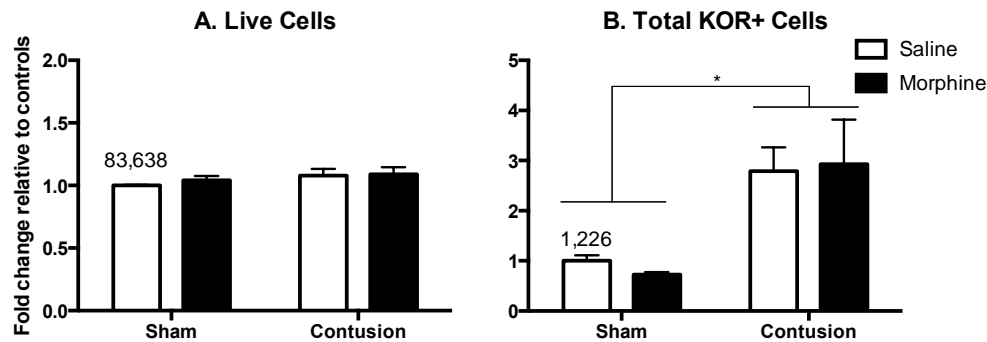
**Figure 58: Microglia / macrophages and co-localization with MOR and CD206.** Surgery significantly affected the total number of microglia/macrophages (A), the number of MOR+ microglia/macrophages (B), and the number MOR+ & CD206+ microglia/macrophages (D) at the site of injury, with no significant effect of drug treatment or interaction. Drug treatment significantly affected CD206+ microglia/macrophages at the site of injury, with no effect of surgery or interaction (C). Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests; #  $p < 0.05$  for planned comparisons; *M* = significant main effect of morphine. Total number of cells in sham + saline group indicated in the first bar.

0.05) or interaction ( $F(1, 20) = 0.84, p > 0.05$ ). On the other hand, when I looked at CD206 expression, I found a significant main effect of drug treatment ( $F(1, 20) = 4.37, p < 0.05$ ) on the number of CD206+ microglia/macrophages, but no effect of surgery ( $F(1, 20) = 2.19, p > 0.05$ ) or interaction ( $F(1, 20) = 0.14, p > 0.05$ ). Finally, when I

looked at the number of microglia/macrophages expressing both, MOR and CD206, statistical analyses uncovered a significant main effect of surgery ( $F(1, 20) = 23.08, p < 0.05$ ), but no effect of drug treatment ( $F(1, 20) = 1.52, p > 0.05$ ) or interaction ( $F(1, 20) = 0.17, p > 0.05$ ).

### Exp. 6C KOR co-localization

As with the MOR, I assessed overall KOR expression and its co-localization on immune cells following SCI and morphine treatment. First, I used forward and side scatter to select live cells in our heterogeneous samples. There were no significant differences in the number of total live cells across the groups (for all,  $p > 0.05$ ; Figure 59).



*Figure 59: KOR+ cells at the injury epicenter.* Injury significantly increased the number of total KOR+ cells at the site of lesion, with no effect of drug administration or interaction. Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests. Total number of cells in sham + saline group indicated in the first bar.

Next, I quantified the total number of KOR-expressing cells at the injury center (this includes immune cells, as well as other cell types such as neurons and astrocytes). Statistical analyses showed a significant increase in total KOR+ cells following injury ( $F(1, 20) = 12.81, p < 0.05$ ), but no effect of drug treatment ( $F(1, 20) = 0.02, p > 0.05$ ) or significant interaction ( $F(1, 20) = 0.14, p > 0.05$ ).

As in the previous experiment, to evaluate KOR expression specifically on immune-competent cells, once again I identified microglia/macrophages using CD11b and IBA1 markers. Corroborating our previous findings (Exp. 6A and Exp. 6B), I found a significant main effect of surgery ( $F(1, 20) = 12.40, p < 0.05$ ) on the number of microglia/macrophages (Figure 60). Drug treatment approached, but did not reach significance ( $F(1, 20) = 2.30, p > 0.05$ ), and there was no significant interaction ( $F(1, 20) = 0.39, p > 0.05$ ). Next, I looked at microglia/macrophages that also expressed KOR and CD206. I found a significant main effect of surgery ( $F(1, 20) = 8.06, p < 0.05$ ) on the number of KOR+ microglia/macrophages, but no effect of drug treatment ( $F(1, 20) = 0.00, p > 0.05$ ) or interaction ( $F(1, 20) = 0.05, p > 0.05$ ). Similarly, when I looked at CD206 expression, I found a significant main effect of surgery ( $F(1, 20) = 31.68, p < 0.05$ ) on the number of CD206+ microglia/macrophages, but no effect of drug treatment ( $F(1, 20) = 2.19, p > 0.05$ ) or interaction ( $F(1, 20) = 0.14, p > 0.05$ ). Finally, when I looked at the number of microglia/macrophages expressing both, KOR and CD206, statistical analyses uncovered a significant main effect of surgery ( $F(1, 20) = 31.57, p < 0.05$ ), but no effect of drug treatment ( $F(1, 20) = 0.002, p > 0.05$ ) or interaction ( $F(1, 20) = 0.029, p > 0.05$ ).

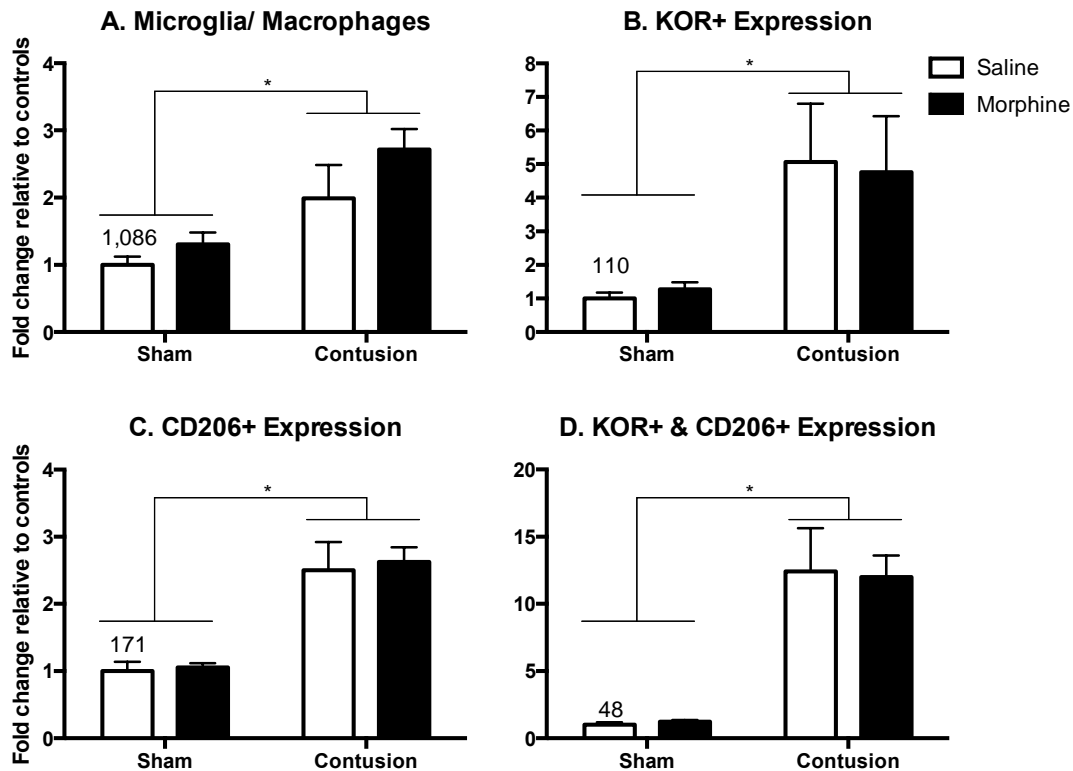
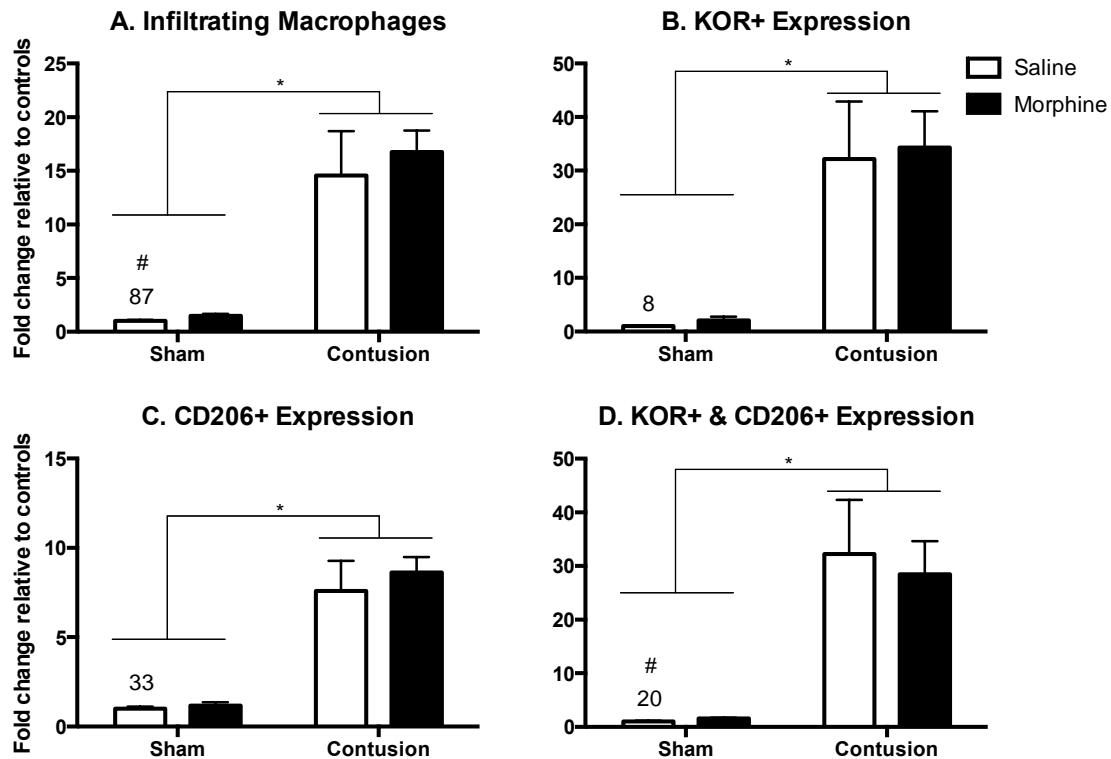


Figure 60: Microglia / macrophages and co-localization with KOR and CD206. Surgery significantly affected the total number of microglia/macrophages (A), KOR+ microglia/macrophages (B), CD206+ microglia/macrophages (C), and KOR+ & CD206+ microglia/macrophages (D) at the site of injury, with no significant effect of drug treatment or interaction. Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests. Total number of cells in sham + saline group indicated in the first bar.

As in experiment 6.A, in these samples I also evaluated the local and peripheral immune response using CD45 expression. The microglia/macrophages previously described were classified into infiltrating macrophages (high CD45 expression) or resident microglia (low CD45 expression), and I assessed their KOR and CD206 expression (Figure 61). I found a significant main effect of surgery ( $F(1, 20) = 32.96$ ,  $p$





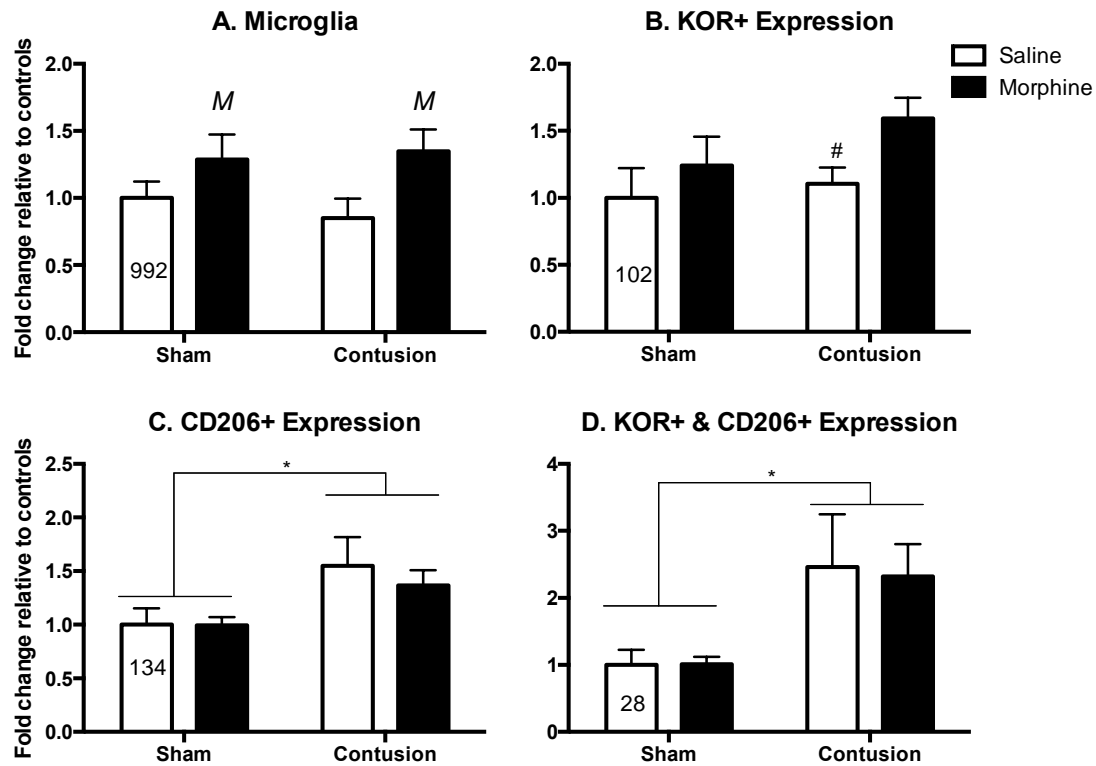
**Figure 61: Infiltrating macrophages and co-localization with KOR and CD206.** Surgery significantly affected the total number of infiltrating macrophages (A), KOR+ macrophages (B), CD206+ macrophages (C), and KOR+ & CD206+ macrophages (D) at the site of injury, with no significant effect of drug treatment or interaction. Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests; # $p < 0.05$  for planned comparisons. Total number of cells in sham + saline group indicated in the first bar.

$< 0.05$ ) on the number of infiltrating macrophages, but no effect of drug treatment ( $F(1, 20) = 0.28$ ,  $p > 0.05$ ) or interaction ( $F(1, 20) = 0.12$ ,  $p > 0.05$ ). Next, I looked at infiltrating macrophages that also expressed KOR. I found a significant main effect of surgery ( $F(1, 20) = 20.86$ ,  $p < 0.05$ ) on the number of KOR+ infiltrating macrophages, but no effect of drug treatment ( $F(1, 20) = 0.05$ ,  $p > 0.05$ ) or interaction ( $F(1, 20) = 0.005$ ,  $p > 0.05$ ). Similarly, when I looked at CD206 expression, I found a significant

main effect of surgery ( $F(1, 20) = 45.27, p < 0.05$ ) on the number of CD206+ infiltrating macrophages, but no effect of drug treatment ( $F(1, 20) = 0.32, p > 0.05$ ) or interaction ( $F(1, 20) = 0.17, p > 0.05$ ). Finally, when I looked at the number of infiltrating macrophages expressing both, KOR and CD206, statistical analyses uncovered a significant main effect of surgery ( $F(1, 20) = 20.25, p < 0.05$ ), but no effect of drug treatment ( $F(1, 20) = 0.002, p > 0.06$ ) or interaction ( $F(1, 20) = 0.12, p > 0.05$ ).

The resident immune cells of the CNS, microglia (low CD45 expression), were also quantified and assessed for their KOR and CD206 expression (Figure 62). As opposed to what I observed with infiltrating macrophages, I found a significant main effect of drug treatment ( $F(1, 20) = 5.24, p < 0.05$ ) on the total number of microglia, but no effect of surgery ( $F(1, 20) = 0.07, p > 0.05$ ) or interaction ( $F(1, 20) = 0.37, p > 0.05$ ). Next, I looked at microglia that also expressed KOR. I found no effect of surgery ( $F(1, 20) = 1.31, p > 0.05$ ) or interaction ( $F(1, 20) = 0.39, p > 0.05$ ), and the effect of drug treatment on the number of KOR+ microglia approached, but did not reach significance ( $F(1, 20) = 3.33, p > 0.05$ ). However, it is important to highlight that planned comparisons showed that our contused subjects treated with morphine had significantly more KOR+ microglia than their saline-treated counterparts. When I looked at CD206 expression, I found a significant main effect of surgery ( $F(1, 20) = 5.86, p < 0.05$ ) on the number of CD206+ microglia, but no effect of drug treatment ( $F(1, 20) = 0.25, p > 0.05$ ) or interaction ( $F(1, 20) = 0.22, p > 0.05$ ). Finally, when I looked at the number of microglia expressing both, KOR and CD206, statistical analyses uncovered a

significant main effect of surgery ( $F(1, 20) = 7.03, p < 0.05$ ), but no effect of drug treatment ( $F(1, 20) = 0.02, p > 0.06$ ) or interaction ( $F(1, 20) = 0.02, p > 0.05$ ).



*Figure 62: Microglia and co-localization with KOR and CD206.* Drug treatment significantly affected the total number of microglia (A). There were no significant effects of surgery or drug treatment on the number of KOR+ microglia (B). Surgery significantly affected the number CD206+ (C) and KOR+ & CD206+ microglia (D) at the site of injury, with no significant effect of drug treatment or interaction. Results shown as Mean  $\pm$  S.E.M. \* $p < 0.05$  for post-hoc tests; # $p < 0.05$  for planned comparisons; M = significant main effect of morphine. Total number of cells in sham + saline group indicated in the first bar.

## Discussion

In these experiments, I used immunohistochemistry and flow cytometry to characterize the cellular environment at the site of lesion 24 hours following morphine administration. First, I conducted a histological assessment of tissue sparing in contused subjects and uninjured controls, treated with morphine or saline (Experiment 5.A). I found that SCI significantly affected lesion size, tissue damage, residual gray matter, and residual white matter, with no added effect of morphine. Next, I used immunohistochemistry to quantify three key cell populations at the lesion site: neurons, astrocytes, and microglia/macrophages (Experiment 5.B). As expected, I found that SCI significantly decreased the number of remaining neurons (NeuN+) and increased the number of microglia/macrophages (CD11b+) across the rostral-caudal extent of the spinal cord. Morphine further decreased the number of remaining neurons, but only caudal to the injury. At this time point, astrocyte (GFAP+) expression was not affected at any level of the spinal cord by either injury or morphine treatment.

For a more in-depth investigation of the opioid-immune interactions that could underlie the adverse effects of morphine, I assessed the role of infiltrating macrophages and microglia, and their expression of MOR and KOR, using flow cytometry. I found that SCI significantly increased the number of infiltrating macrophages, while morphine significantly increased the number of microglia regardless of injury (Experiment 6.A). The number of microglia/macrophages expressing the MOR was also significantly increased by SCI (Experiment 6.B), as was the total number of KOR+ cells and KOR+ microglia/macrophages (Experiment 6.C). Interestingly, contused subjects treated with

morphine also showed significantly higher numbers of KOR+ microglia than their saline-treated counterparts.

As expected, our results showed that microglia/macrophages are increased at the site of lesion following SCI. More interesting, however, was the significant effect of morphine on these cells types, increasing their presence in the spinal cord regardless of injury. Furthermore, when assessing immune polarization, I also observed trends suggesting that morphine may bias microglia toward an M1 phenotype. Although this was effect did not reach significance, extensive in vitro and in vivo evidence suggests that opioid administration results in glial activation and the release of pro-inflammatory cytokines (Cui et al., 2006; Hutchinson et al., 2008a; Johnston et al., 2004; Raghavendra et al., 2002; Raghavendra et al., 2004; Song & Zhao, 2001). For instance, lumbar dorsal spinal cord sections show significant increases in the release of IL-1 $\beta$ , IL-6, fractalkine, GRO/KC, MIP-1 $\alpha$ , MCP-1 and TNF- $\alpha$  following 180 minutes of incubation with 100  $\mu$ M morphine compared to media alone (Hutchinson et al., 2008a). In vivo, increases in IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and TNF- $\alpha$  have also been demonstrated after 7 days of intrathecal morphine (Hutchinson et al., 2008a). It is clear that opioids can modulate the immune response from an anti-inflammatory (M2) to a pro-inflammatory and neurotoxic (M1) phenotype.

Supporting an immunomodulatory role of the classic opioid receptors, here I presented evidence of MOR and KOR expression on immune cells, a finding that is supported by the literature (Bidlack et al., 2006; McCarthy et al., 2001; Rogers & Peterson, 2003; Sharp, 2006). I also showed that following SCI, the total number of

KOR+ cells, but not MOR+ cells, is increased at the lesion site. This is also consistent with the literature. For instance, a study using selective radioligands for the classic opioid receptors showed a significant increase in KOR binding, but not the other receptor types, on spinal tissue collected 2 hours, 24 hours, and 1 week after a T10 contusion (Krumins & Faden, 1986). Surprisingly, I also observed a significant increase in KOR+ microglia at the lesion site of contused subjects treated with morphine, when compared to their saline-treated counterparts. Notably, this may increase the vulnerability of these cells to dynorphin, an endogenous KOR ligand that is significantly elevated in the spinal cord following trauma, and which has been implicated in the pathophysiology of SCI (Cox et al., 1985; Faden et al., 1985a; Faden et al., 1985b; Przewłocki et al., 1988; Tachibana et al., 1998).

Aside from the immune cells investigated in this study, the significant increase in total KOR+ cells following SCI suggests that other cell populations are also vulnerable to the effects of endogenous and exogenous KOR ligands. For instance, Adjan et al. (2007) measured caspase-3 activity at the injury site in wild-type and prodynorphin knockout mice following an experimental spinal contusion. They found that not only was caspase-3 significantly decreased in tissue homogenates from prodynorphin knockout mice, but astrocytes and oligodendrocytes in these subjects also expressed significantly lower levels of active caspase-3 hours after injury (Adjan et al., 2007). Aside from directly engaging apoptotic mechanisms following SCI, KOR signaling may also contribute to secondary injury through aberrant glial activation. A role for the KOR in glial activation and proliferation following trauma is supported by evidence from

other injury models. Following partial sciatic nerve ligation, KOR immunoreactivity is significantly increased in dorsal horn GABAergic neurons and astrocytes, ipsilateral and contralateral to the injury (Xu et al., 2004). Importantly, KOR activation appears to play a direct role in the astrocytic response to injury. As reported by Xu et al. (2007), mice with a dynorphin knockout, a KOR knockout, or pretreated with norBNI, lacked the marked increase in GFAP immunoreactivity observed 1-week post-nerve injury in wild-type controls. In vitro, cultured astrocytes treated with the KOR agonist U50,488 showed a significantly increased proliferation rate, and this effect was blocked by norBNI (Xu et al., 2007). Altogether, the release of pro-inflammatory factors from immune and glial cells vulnerable to KOR ligands could contribute to the neurotoxic environment following SCI. This could explain the significant decrease in neurons observed caudal to the injury in contused subjects treated with morphine, when compared to saline-treated controls (Experiment 5.B).

In sum, the KOR also appears to play an important role in the mechanisms leading to the adverse effects of morphine administration following SCI. I hypothesize that these effects are the result of an opioid-induced exacerbation of the inflammatory response intrinsic to SCI. *In vitro* and *in vivo* evidence suggests that opioid administration results in the activation of glial cells and the release of pro-inflammatory cytokines (Cui et al., 2006; Hutchinson et al., 2008a; Johnston et al., 2004; Raghavendra et al., 2002; Raghavendra et al., 2004; Song & Zhao, 2001). While non-classic opioid receptor signaling has been implicated in these opioid-immune interactions (Hutchinson et al., 2010b; Wang et al., 2012), our findings point to a previously overlooked role of

the KOR. Based on the results of this study, I posit that morphine's adverse effects may result from activation of KORs on glial cells. The synergistic effects of immune cell activation and KOR-mediated gliopathy could explain the decreased locomotor recovery, increased nociceptive reactivity, and decreased tissue sparing observed in our rodent model when morphine is administered following SCI (Hook et al., 2007; Hook et al., 2009; Hook et al., 2011). These results underscore the need for further studies targeting the cell-specific effects of opioid administration, and other medications, following SCI. Overall, however, these data suggest that KOR antagonists and glial inhibitors may be viable adjuvants to morphine, reducing the adverse long-term consequences of opioid administration in the acute phase of SCI.



## CHAPTER VI

### GENERAL DISCUSSION AND CONCLUSIONS

The experiments presented here investigated the mechanisms underlying the adverse effects of morphine, the prototypical opioid, in a rodent model of SCI. Commensurate with our previous studies, I found that morphine administered in the acute phase of SCI decreased locomotor recovery and increased the size of the spinal lesion. After an evaluation of the classic and non-classic opioid receptors, I found that the  $\kappa$ -opioid receptor (KOR) system plays a significant role in the morphine-induced attenuation of function. I also found that glial activation is critical to morphine's adverse effects. Lastly, I observed that morphine significantly increases the microglial response following SCI, and that this mechanism may be mediated by co-localization with KORs. Our data suggests that SCI increases the expression of classic opioid receptor subtypes on immune cells. In the following sections I will discuss the effects of selective opioid receptor activation on recovery after SCI, and show that injury-induced changes in opioid-immune interactions may significantly change the consequences of exogenous opioids administered for pain management after injury.

#### **Contribution of the opioid receptors**

In these experiments, I used selective opioid receptor agonists and antagonists to assess the contribution of the classic and non-classic opioid receptors to the adverse effects of morphine following SCI. Our main finding was that the KOR significantly contributes to morphine's adverse effects. The selective KOR-agonist GR89696

significantly undermined recovery of locomotor function, increased mechanical reactivity, and decreased gray matter sparing after SCI. This was observed at a dose 32 times lower than the 0.32  $\mu\text{mol}$  (90  $\mu\text{g}$ ) dose of morphine previously used in our studies (Hook et al., 2009; Hook et al., 2011). The powerful effects of selective KOR activation are consistent with previous literature. In fact, it has been reported that dynorphin and dynorphin-related peptides can induce paraplegia even when administered to the intact spinal cord (Faden & Jacobs, 1983; Herman & Goldstein, 1985). Further supporting a KOR-mediated mechanism, pretreatment with the KOR-antagonist norBNI significantly reduced morphine's adverse effects. Others have also shown that norBNI limits the paralytic effects of dynorphin (Faden, 1990), and can improve functional outcomes in rodent models of spinal and brain injury (Faden, 1990; Faden et al., 1987; Vink et al., 1991). Although I did not find a therapeutic effect of norBNI per se, our data suggests that KOR activation is sufficient and necessary for the morphine-induced attenuation of function following SCI.

Contrary to our expectations, however, I found that selective  $\mu$ -opioid receptor (MOR) activation using DAMGO does not replicate morphine's adverse effects on locomotor function. Although the literature suggests that MOR activation may be linked to excitotoxicity (L. Chen & Huang, 1992; L. Chen & Marine, 1991; Mao et al., 1995; Mayer et al., 1995), I did not observe behavioral changes suggesting increased cell death at the lesion site. Opioid-induced excitotoxicity, however, has been primarily reported following prolonged morphine treatment (Mao et al., 1994; Mao et al., 1995; Mao et al., 2002a, 2002b; Marek et al., 1991; Mayer et al., 1995; Trujillo & Akil, 1991; L. Yang et

al., 2008). In our studies, only a single opioid administration was given; therefore, it is possible that this did not induce the changes in MOR activity necessary to produce excitotoxicity. Alternatively, the null effects of MOR activation in our studies could also reflect differences in the rate of elimination between DAMGO and morphine. Indeed, the half-life of DAMGO is approximately 30 minutes (Szeto et al., 2001), while the half-life of morphine is between 1.5 – 4.5 hours (Paul, 2015). To address this, I challenged morphine with CTOP, a MOR antagonist. Surprisingly, I found that CTOP did not block morphine's adverse effects, but instead promoted the development of paradoxical pain in the chronic stage of SCI. In comparison to controls treated with saline only, subjects treated with CTOP showed increased mechanical allodynia (vocal reactivity to von Frey stimulation) 21 days post-injury. This may reflect a role of MOR activation in endogenous pain control following neurotrauma. This finding also underscores the necessity for mitigation of pain in the early phase of injury. As suggested by others (J. Katz et al., 1992; Møiniche et al., 2002; Pergolizzi et al., 2014; Woolf & Chong, 1993), effective management of acute pain appears to be necessary to prevent the chronification of SCI-related pain.

Aside from the MOR and KOR, I also hypothesized that morphine may exert its negative effects through activation of non-classic opioid receptors found on immune-competent cells, such as TLR4 (Wang et al., 2012). Binding to these receptors leads to glial activation, and the subsequent release of pro-inflammatory cytokines in the spinal cord, which is associated with decreased opioid analgesia and pain facilitation (Hutchinson et al., 2007; Hutchinson et al., 2008a; Hutchinson et al., 2011). However,

treatment with the TLR4 agonist LPS or [+]- morphine did not replicate morphine's adverse effects on recovery of function. This was not due to defective LPS, as the functionality of the drug was verified in a separate experiment. Instead, it is possible that following SCI, TLR activation may approach a ceiling effect, due to the highly inflammatory nature of the injury per se. Indeed, although there were no effects of LPS treatment, pre-treatment with the TLR4 antagonist LPS-RS blocked the adverse effects of morphine. Similarly, in a previous study, pre-treatment with an IL-1 receptor antagonist also prevented morphine's adverse effects (Hook et al., 2011). The IL-1 and TLR4 receptors engage highly congruent signaling pathways: upon binding, adaptor proteins are recruited that activate downstream kinases and transcription factors, such as NF- $\kappa$ B, ultimately leading to the production of pro-inflammatory cytokines and chemokines (Cohen, 2014). Importantly, in our study, treatment with minocycline also protected against morphine's unwanted side effects. Minocycline is a tetracycline antibiotic shown to promote neuroprotection by inhibiting glial activation and proliferation, and reducing inflammation (Ledeboer et al., 2005; Plane et al., 2010; Tikka et al., 2001). Together, the data presented here suggest that the immune response following SCI is intimately involved in morphine's adverse effects.

### **Opioid-immune interactions**

#### *SCI alters opioid receptor expression*

SCI not only increases the number of immune cells present in the spinal cord, it also appears to increase the expression of the classic opioid receptors on these cells. Using flow cytometry, I found that SCI significantly increased the number of infiltrating

macrophages at the lesion site by day 3 post-injury. This coincides with the time course of infiltration previously described in the literature (Beck et al., 2010; Popovich et al., 1997). Interestingly, I also found that injury significantly upregulates total KOR expression, but not MOR expression, in the spinal cord. The total number of KOR<sup>+</sup> cells was approximately three times higher in SCI subjects compared to uninjured controls. Others have also found increased expression of KOR, but not the other receptor types, on spinal tissue collected 2 hours, 24 hours, and 1 week after a T10 contusion (Krumins & Faden, 1986). Unfortunately, in our experiments, I further pursued KOR co-localization only in microglia and macrophages. However, I suspect that increased KOR expression also occurred in other cell types following SCI. Indeed, the literature shows that aside from neurons and microglia, other cells such as astrocytes, oligodendrocytes, and peripheral immune cells also express classic opioid receptors (Belcheva et al., 2005; Bidlack et al., 2006; Bruchas et al., 2006; Chao et al., 1997; Eriksson et al., 1993; Gurwell et al., 1996; Hauser et al., 1996; McLennan et al., 2008; Ruzicka et al., 1995; Sharp, 2006; Stiene-Martin et al., 2001; Stiene-Martin et al., 1998; Xu et al., 2007).

I hypothesize that the increased opioid receptor expression may be associated with cytokines and chemokines present in the acutely injured cellular environment. Indeed, co-administration of IL-1 $\alpha$  and IL-1 $\beta$  has been shown to increase MOR expression in neural microvascular endothelial cell cultures (Vidal et al., 1998). Similarly, treatment with IL-1 $\beta$  for 24 hours can alter MOR and KOR mRNA expression in tissue cultures from the rat striatum, cerebellum, hippocampus, hypothalamus, and cortex (Ruzicka & Akil, 1997; Ruzicka et al., 1996). Cytokines appear to induce

upregulation of opioid receptors in immune and glial cells through the activation of transcription factors. For instance, IL-6-mediated upregulation of MOR expression is dependent on STAT (signal transducer and activator of transcription) 1 and STAT3 binding to the promoter region of the opioid receptor gene (Börner et al., 2004). This may explain the reduced opioid analgesia and decreased MOR expression observed in IL-6 knockout mice (Bianchi et al., 1999). Stimulation with TNF- $\alpha$  has also been shown to induce MOR gene transcription in primary human T lymphocytes, Raji B cells, U937 monocytes, primary human polymorphonuclear leukocytes and mature dendritic cells through activation of NF- $\kappa$ B (Kraus et al., 2003). Altogether, this suggests that pro-inflammatory cytokines play an important role in opioid receptor regulation following SCI, which may significantly alter the effects of opioid analgesics when administered in a pathophysiological context.

#### *Morphine increases microglia at the injury site*

Further supporting an immunomodulatory role of opioids, I found that morphine significantly increased the number of microglia present in the spinal cord regardless of injury. This may result from opioid-induced increases in cell recruitment. For example, in microglia cultures, morphine treatment promotes morphological changes, activation, and chemotaxis- effects that are blocked by naloxone (Horvath & DeLeo, 2009; Takayama & Ueda, 2005; Tsai et al., 2015). Studies suggest that morphine may induce microglial migration via a mechanism involving the ionotropic purinergic receptor P2X4, which has also been implicated in aberrant glial activation and pathological pain (Biber et al., 2011; Horvath & DeLeo, 2009; Inoue et al., 2004; Tsuda et al., 2003).

Furthermore, opioid-induced proliferation has been observed in hippocampal neural progenitors, vascular endothelial cells, and peripheral blood mononuclear cells (Hutchinson et al., 2004; Leo et al., 2009; Persson et al., 2003). Therefore, it is possible that the increase in microglia that I observed could also be due to increased proliferation stimulated by morphine treatment. Irrespective of whether the increase in microglia resulted from increased recruitment or proliferation, the overall effect could lead to an elevation in the production and release of pro-inflammatory cytokines. Indeed, increases in IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and TNF- $\alpha$  have been demonstrated after 7 days of intrathecal morphine (Hutchinson et al., 2008a). In the context of SCI, opioids could act synergistically with the innate immune response to exacerbate inflammation, increasing the neurotoxic environment at the lesion site.

Our experiments also showed that microglia and macrophages express classic opioid receptors after SCI. Contused subjects showed 5 times the number of MOR+ and KOR+ microglia/macrophages at the lesion compared to sham controls. Additionally, contused subjects treated with morphine showed significantly more KOR+ microglia than their saline-treated counterparts. This finding supports the immunomodulatory role of opioids that has been previously described in the literature (Bidlack et al., 2006; McCarthy et al., 2001; Rogers & Peterson, 2003; Sharp, 2006). More importantly, however, this may also provide a mechanism of action for morphine's adverse effects. As shown in our pharmacological studies, KOR signaling and glial activation are necessary for morphine-induced attenuation of locomotor recovery. Increased KOR expression may leave microglia more vulnerable to dynorphin, an endogenous KOR

ligand that is significantly elevated in the spinal cord following trauma, and which has been implicated in the pathophysiology of SCI (Cox et al., 1985; Faden et al., 1985a; Faden et al., 1985b; Przewłocki et al., 1988; Tachibana et al., 1998). Furthermore, the significant increase in total KOR+ cells following SCI suggests that other cell populations are also vulnerable to the effects of endogenous and exogenous KOR ligands. For instance, KOR signaling has been implicated in spinal astrocyte activation and proliferation following nerve injury via a p38 MAPK-mediated mechanism (Xu et al., 2007; Xu et al., 2004). KOR signaling may also be associated with Caspase-3 activation, since dynorphin knockout decreases astrocytes and oligodendrocyte apoptosis in a mouse model of SCI (Adjan et al., 2007). Overall, the evidence presented here suggests that neurotrauma may significantly alter the effects of exogenous opioids by changing the cellular expression of the opioid receptor subtypes.

### **Clinical significance**

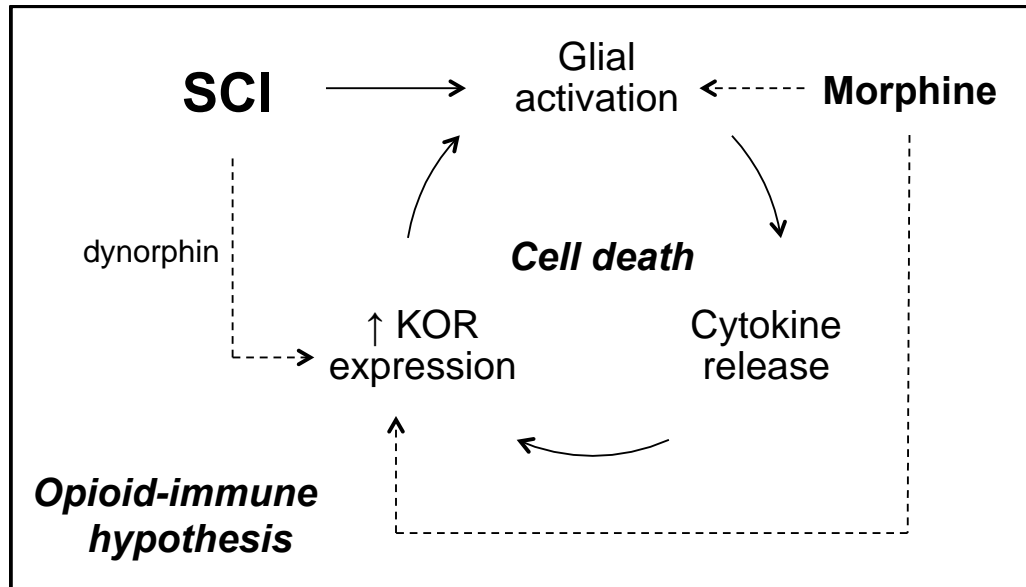
#### *Locomotor recovery*

Studies from our laboratory have consistently shown that opioids are contraindicated following SCI (Hook et al., 2007; Hook et al., 2009; Hook et al., 2011; Hook et al., 2016). In our model, morphine administered in the acute phase of SCI leads to significant attenuation of functional recovery. These effects appear to be the result of opioid-induced cell death. Indeed, in the experiments presented here, even a single administration of 90 µg of intrathecal morphine, or an equivalent dose of a KOR agonist, decreased residual tissue at the site of lesion 21 days post-injury in comparison to vehicle-treated controls. Using immunohistochemistry, I also found that as early as 24



hours following morphine treatment, contused subjects treated with morphine show decreased neuron expression caudal to the injury epicenter than their saline-treated counterparts. Although at this time point I did not find any additional effects of morphine on astrocytes or macrophages, I know from our previous work that 7 days of morphine treatment completely depletes neurons and astrocytes at the lesion center and increases markers of neurodegeneration (Hook et al., 2016). Understanding the molecular mechanisms underlying morphine's adverse effects is essential to improve the safety and efficacy of clinically-used opioids. I propose that morphine attenuates recovery of function by exacerbating the immune response following SCI via a KOR-mediated mechanism (described in Figure 63).

The mechanism is initiated by SCI, as damage resulting from the primary injury triggers the innate immune response, leading to neutrophil and macrophage infiltration and activation of immune-competent glial cells in the spinal cord. In microglia, TLRs and purinergic receptors engage intracellular signaling mechanisms that activate transcription factors like the MAPKs and NF- $\kappa$ B. This leads to the production and release of pro-inflammatory cytokines and chemokines, including IL-1 $\beta$ , IL-6, fractalkine, GRO/KC, MIP-1 $\alpha$ , MCP-1 and TNF- $\alpha$ , along with other excitatory factors. Subsequently, the release of pro-inflammatory cytokines and chemokines from microglia promotes the recruitment of other immune cells, as well as glial activation and proliferation. The release of pro-inflammatory cytokines may also lead to upregulated opioid receptor expression in microglia, astrocytes, and other cells, making them vulnerable to endogenous dynorphin and exogenous opioids.



*Figure 63: Proposed mechanism of morphine-induced cell death following SCI.* Injury to the spinal cord initiates an innate immune response leading to microglia/macrophage activation, pro-inflammatory cytokine release, and a subsequent increase in KOR expression. Upon administration, morphine binds to KORs on immune-competent cells, further increasing glial activation, chemotaxis, and proliferation. This exacerbates the neurotoxic environment, increasing cell death, and attenuating recovery of function.

Under these conditions, morphine can act synergistically with the immune response to exacerbate secondary damage. Upon administration, morphine binds to KORs expressed on microglia and other immune-competent cells. Subsequently, the KOR can signal through its G-protein, ultimately activating the MAPKs, which then regulate cell proliferation and cytokine release. This activates a positive feedback loop, further activating immune-competent cells and prolonging cytokine release. The sustained release of pro-inflammatory cytokines from immune competent cells, in

combination with other SCI mechanisms, including excitatory neurotransmitter release and downregulation of glutamate transporters, exacerbates the neurotoxic environment.

### *Pain management*

While our focus has been on the morphine-induced attenuation of locomotor recovery after SCI, changes in opioid receptor expression will also affect the clinical management of other conditions associated with SCI, including pain, addiction, and depression. It is well-established that injury and inflammation decrease the analgesic efficacy of opioids. As previously discussed, the inflammatory response produces an excitatory cellular environment that sensitizes neurons, diminishing the net inhibitory function of opioid analgesics. Additionally, opioids per se can increase glial activation and the production of pro-inflammatory cytokines, counteracting their own antinociceptive effects (Hutchinson et al., 2008a; Hutchinson et al., 2010b; Lewis et al., 2010). Changes in MOR expression following injury may significantly contribute to these effects. In our studies, although I observed no change in the total number of MOR+ cells, there was a significant increase in MOR+ microglia/macrophages after SCI. This suggests that, following injury, more MORs may be located on immune-competent cells than remaining neurons. Importantly, this could induce a shift in the overall effect of opioid administration, from anti-nociceptive to pro-inflammatory and even neurotoxic.

Based on this, it might appear that opioids are not suitable analgesics for the treatment of neuropathic or inflammatory pain. However, our findings suggest that anti-inflammatories may be used as adjuvants to opioid administration, to improve the safety and analgesic efficacy of these drugs. In animal models of nerve injury, for example,

treatment with glial inhibitors like minocycline and pentoxifylline significantly potentiates morphine analgesia (Mika et al., 2007) and decreases tolerance (Cui et al., 2008; Mika et al., 2009). Interestingly, blocking glial activation alone also attenuates the development of allodynia and hyperalgesia after injury (Chang & Waxman, 2010; Ledebøer et al., 2005; Mika et al., 2007), suggesting that the combination of opioids and glial inhibitors may be used to manage and treat neuropathic pain in the clinic. Furthermore, evidence also suggests that glial inhibitors, such as minocycline, may even provide neuroprotection (Mejia et al., 2001; Stirling et al., 2004). Our current research suggests that minocycline effectively blocks the adverse effects of morphine following SCI. Indeed, minocycline and similar drugs are currently being evaluated in a variety of clinical trials as treatments for neurodegenerative conditions like Huntington disease and Parkinson disease (Kim & Suh, 2009; Plane et al., 2010). Although still under investigation, our studies and those of others, suggest that glial inhibitors are promising therapeutic strategies for neurotrauma and neurodegenerative disease.

### *Addiction*

Concerns regarding the possibility for addiction greatly complicate pain treatment in the clinic. It is well-known that under physiological conditions, opioids act as strong reinforcers, often leading to drug abuse. Chronic pain patients may be particularly vulnerable to developing addiction since the repeated administration of opioid drugs often leads to tolerance and a need for escalating doses. The mechanisms underlying opioid addiction, however, appear to be altered under conditions leading to the development of chronic pain. For example, it appears that following neurotrauma,

the rewarding and addictive properties of opioids are decreased. In a study published in 2013 by Wade and colleagues (Wade et al., 2013), fentanyl self-administration in mice was investigated under three different pain models: CFA-induced inflammation, spinal nerve ligation, and chronic chemotherapeutic-induced neuropathy. In all conditions, fentanyl reduced or failed to support self-administration compared to controls, although lever-pressing for a food reinforcer was unaffected (Wade et al., 2013). This suggests that the decreased operant behavior during pathological pain was probably due to decreases in the rewarding property of the opioid, and not due to motor or learning deficits. Others have shown that rats undergoing formalin or carrageenan-induced inflammation do not develop a preference for a morphine-paired context (Suzuki et al., 1996). Similarly, mice undergoing experimental cancer pain do not develop morphine-induced place preference (Betourne et al., 2008), and spinally-injured rats self-administer significantly less morphine than their sham-counterparts early after SCI (Woller et al., 2014; Woller et al., 2012).

As discussed for pain and locomotor recovery, these changes may reflect altered opioid receptor expression following injury to the nervous system. Converging lines of evidence suggest that inflammation and injury may reduce the rewarding properties of opioids by changing opioid receptor expression and function. Normally, opioids exert their rewarding properties by activating cells in the ventral tegmental area (VTA), which send dopaminergic signals to the nucleus accumbens. Decreased opioid receptor expression in these areas due to inflammation and injury, however, may inhibit the opioid-induced activity in this reward center observed under physiological conditions.

Indeed, sciatic nerve ligation suppresses increased dopamine release in the nucleus accumbens normally observed after morphine administration in rodents (Niikura et al., 2008; Ozaki et al., 2002). Furthermore, opioid-facilitation of the rewarding effects of VTA electrical stimulation is also suppressed in rats after a spinal nerve ligation (Ewan & Martin, 2011).

Increased expression of specific opioid receptor subtypes due to the release of pro-inflammatory cytokines may also play a role in decreased reward. The KOR system appears to be a particularly important player in these effects. For example, treatment with KOR agonists results in decreased morphine and cocaine intake in a model of rodent self-administration (Glick et al., 1995; Schenk et al., 1999). KOR activation may produce this effect by reducing extracellular dopamine in the nucleus accumbens (Maisonneuve et al., 1994). Conversely, nor-BNI treatment has been shown to reverse the attenuation of morphine-induced place preference in rats injected with formalin (Suzuki et al., 1998), probably by countering KOR-driven inhibition of dopamine turnover in limbic regions (Narita et al., 2005). Similar mechanisms might underlie decreased morphine self-administration observed early after SCI in rodents (Woller et al., 2014; Woller et al., 2012). By binding to upregulated supraspinal KORs, morphine may counteractively decrease, rather than increase, opioid reward. However, as inflammation subsides, KOR expression may return to baseline levels, no longer suppressing addiction. This might explain why, although contused rodents self-administer significantly less morphine than their sham-counterparts in the first 7 days

after injury, the differences between injured subjects and sham controls disappear if self-administration is started on days 14 or 35 after SCI.

Altogether, these findings have many important implications for the addictive potential of opioid drugs used after damage to the nervous system. Mainly, they suggest that altered opioid receptor expression in supraspinal regions may decrease the rewarding properties of opioids after injury. Nevertheless, this does not mean that the potential for addiction is completely abolished under neuropathic pain conditions. Increased dosage, for example, may overcome KOR-mediated suppression of dopamine turnover. Additionally, opiates that have partial KOR antagonist properties, like buprenorphine, may even increase reward. Ultimately, a better understanding of altered opioid receptor expression after injury to the nervous system will inform clinicians on the addictive potential of specific opioid analgesics, improving the safe use of these powerful drugs.

### *Depression*

Not surprisingly, psychological well-being is also significantly affected following neurotrauma. Within the spinally-injured alone, the prevalence of major depression has been estimated to be within 15-23% (Bombardier et al., 2004), compared to 6.6% in the general U.S. population (Kessler et al., 2003). Importantly, decreased psychological health in injured patients has been associated with numerous negative consequences, including decreased quality of life, decreased health, and even increased mortality (Krause et al., 2009; Krause et al., 2008). This underscores the importance of ensuring the mental, as well as the physical, well-being of patients following injury.

Increasing research suggests that targeting the immune system may be a viable strategy for the treatment of depression following injury to the nervous system. The inflammatory response has been strongly linked with the development of depression and other psychological disorders (Dantzer et al., 2008; Miller et al., 2009). For instance, elevated levels of pro-inflammatory cytokines, chemokines, and other factors have been observed in the peripheral blood circulation and in the cerebrospinal fluid of both healthy and sick patients exhibiting symptoms of depression (Dowlati et al., 2010; Howren et al., 2009; Lanquillon et al., 2000; Levine et al., 1999; Owen et al., 2001). Cytokine-induced depression has also been observed in patients undergoing immunotherapy with interferon (IFN)- $\alpha$  for the treatment of cancer and other diseases (Capuron & Ravaud, 1999; H. Miyaoka et al., 1999; Valentine et al., 1998). In animal models of spinal cord and traumatic brain injury, aberrant glial activation resulting from the injury may also potentiate depressive-like behaviors (Fenn et al., 2014; Luedtke et al., 2014; Wu et al., 2014). Furthermore, there is also data suggesting that anti-inflammatory drugs possess anti-depressive qualities, and should be used as adjuncts to traditional therapies for mood disorders (Akhondzadeh et al., 2009; Guo et al., 2009; T. Miyaoka et al., 2012; Molina-Hernández et al., 2008; Müller et al., 2006).

Cytokines may influence the expression of depressive-like behaviors through a variety of pathophysiological mechanisms. For instance, cytokines may target the monoamines (C. L. Raison et al., 2009; C. L. Raison et al., 2010b). Indeed, altered synthesis, release, and re-uptake of neurotransmitters like serotonin, norepinephrine, and dopamine is a well-known underlying cause of depression in many patients. Cytokines



can also interact with the hypothalamic-pituitary-adrenal (HPA) axis, dysregulating the secretion of hormones that play an important role in the response to stress (Capuron et al., 2003; Kaestner et al., 2005; C. Raison et al., 2010a). Lastly, inflammation may also influence the emergence of depression and other mood disorders by modulating neural plasticity in the brain. Neurodegeneration has been linked with depression in some disorders of the nervous system, like Parkinson's and Alzheimer's (Förstl et al., 1992; Pålhagen et al., 2008; Zubenko & Moossy, 1988). Similarly, cytokines may increase neuronal loss through increased excitotoxicity and apoptosis.

Additionally, cytokines may also play a role in the development of depression by altering opioid receptor expression in the brain after injury. As previously discussed, cytokines can modulate opioid receptor expression. Opioids are well-known mood regulators, causing both euphoric and dysphoric effects. This suggests that opioid receptor activity may play a critical role in affective disorders. Indeed, experimental data suggests that this is the case. For example, mice with a genetic knockout for the MOR or DOR show anxiogenic and depressive-like profiles, although this phenotype is not observed after deletion of the KOR gene (Filliol et al., 2000). The DOR appears to have a particularly strong therapeutic potential. Endogenous activation of DOR, or administration of selective agonists, results in anxiolytic and anti-depressant effects in preclinical models (Jutkiewicz, 2006; Nieto et al., 2005; Perrine et al., 2006; Saitoh et al., 2004). On the other hand, KOR signaling appears to induce negative emotional states. Administration of salvinorin A, a KOR agonist, induces depressive-like behavior in rats (Carlezon et al., 2006), while administration of antagonists, like nor-BNI, have

been shown to decrease immobility time in the forced swim test in a dose-dependent fashion (Mague et al., 2003).

Importantly, opioid treatment alone may significantly affect psychological well-being. Retrospective studies have found that the duration and dose of prescription opioids is strongly correlated with increased risk of depression, even after controlling for pain and other confounding variables (Scherrer et al., 2016; Scherrer et al., 2015; Scherrer et al., 2014). In the context of altered opioid receptor expression following injury, the effects of exogenous opioids on mood may be greatly increased. Research suggests that the ultimate depressant or anti-depressant effects of the analgesic prescribed will be largely driven by actions at specific opioid receptor subtypes (Lutz & Kieffer, 2013b). For example, as with addiction, opioid treatment after SCI may potentiate depression by activating upregulated KORs. This suggests that, for SCI patients about to begin an opioid regimen, treatment with a drug that has known antagonistic properties at the KOR would prevent affective dysregulation. Alternatively, a KOR agonist may produce dysphoria and even exacerbate anxiety and stress. Once again, the significant effects of opioids on psychological wellbeing underscore the importance of understanding the changes in opioid receptor expression after injury to the nervous system.

## **Conclusions**

Opioids, long considered the golden standard of analgesic efficacy, remain one of the most powerful tools available to clinicians for the treatment of acute and chronic pain. This is reflected in the steep increase in opioid use that has been observed in recent

years. For instance, opioid prescriptions in the United States nearly tripled in the past 25 years, skyrocketing from approximately 76 million in 1991, to nearly 207 million in 2013 (National Institute of Drug Abuse (NIDA), 2014). Importantly, this alarming increase in opioid consumption has also lead to rising concerns over the safety of these medications. As I have already discussed, neurotrauma patients may be particularly vulnerable to the negative side effects of these drugs.

Here, I proposed one central idea: the pathophysiological context of an injury alters the function of opioids, decreasing their analgesic efficacy and safety. I reviewed evidence from the literature suggesting that opioids and SCI may act synergistically to exacerbate excitotoxicity, apoptosis, and inflammation. I also presented data from our own studies showing that opioid-immune interactions via a KOR-mediated mechanism may underlie the adverse effects of morphine on recovery of function following SCI. Lastly, I discussed the clinical implications of opioids in a system with altered opioid receptor expression, focusing specifically on locomotor recovery, pain management, addiction, and depression.

Ultimately, evidence from the literature and our findings point to a crucial fact: we need to increase our understanding of opioid receptor expression and function in the injured nervous system in order to improve the safety and efficacy of opioid analgesics. Studies rarely differentiate among the variety of cell types that may show altered opioid receptor expression, usually limiting their assessment to a macroscopic approach. Our data suggest that in order to properly predict how opioid function will change after injury, we need to know how receptor expression will be altered in specific cell

populations. For example, upregulated opioid receptor expression on microglia versus neurons would be expected to affect opioid-induced analgesia in significantly different ways. The time course of altered expression also needs to be further explored, as this will inform clinicians on windows of vulnerability when opioids should be avoided. In SCI, for example, opioid-induced attenuated recovery is only observed if morphine is administered in the acute phase of injury. This may reflect opioid receptor changes that parallel the inflammatory response, and that should be taken into account before beginning an opioid regimen.

Similarly, our understanding of the molecular mechanisms underlying opioid receptor signaling must also expand to improve our use of opiates in the clinic. The evidence suggests that opioid receptors show type-specific, agonist-specific, and oligomerization differences, and all of these factors contribute to opioid signaling. Moreover, ligand-dependent conformational changes can also affect the formation of intracellular signaling complexes, which can drastically change important cell functions depending on the cascade activated. Finally, receptor-receptor interactions (also known as receptor oligomerization) also greatly affect opioid signaling. The MOR and DOR, for instance, can form heterodimers that exhibit synergistic effects upon activation (Gomes et al., 2000).

Clearly, preventing the unwanted effects of opioid administration following neurotrauma is not as easy as blocking one opioid receptor subtype. Thus, one might be tempted to remove opioids from the list of analgesics currently used for the treatment of pain following injury to the nervous system. However, denying patients suffering from

debilitating chronic pain conditions a medication that could relieve their pain, and significantly improve their quality of life, is simply not an option. Instead, functional selectivity of opioids could be achieved by targeting all of the different factors that influence opioid signaling described here. Alternatively, neuroprotective drugs could also be used as adjuncts to inhibit opioid-induced secondary damage. Overall, the experiments presented here underscore the need for further evaluation of opioid-receptor signaling in the pathophysiological context in which they are applied.

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